Diagnosis and Treatment of Unconjugated Hyperbilirubinemia

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Diagnosis and Treatment of Unconjugated Hyperbilirubinemia

Experimental and Clinical Aspects

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"If you are going to try, go all the way. Otherwise don't even start." - Charles Bukowski

Paranimfen: Floris Haijer
Nienke Touw

CONTENTS

Chapter 1

GENERAL INTRODUCTION

1. BILIRUBIN

Bilirubin is a yellow-orange colored bile pigment. The term *bilirubin* is derived from the Latin words for bile (*bilis*) and red (*rubor*). Bilirubin can accumulate in the blood, which is called hyperbilirubinemia. It can also accumulate in other tissues, where it can cause a yellow discoloration of *e.g.* the skin or the sclerae, referred to as jaundice or icterus. Most dangerous is the accumulation of bilirubin in the brain, which may lead to permanent neurological damage. Accumulation of bilirubin in the body has many causes but, eventually, results from an imbalance between bilirubin production and its disposal *via* either metabolism and/or excretion. This thesis focuses on several new strategies for treatment of acute severe (unconjugated) hyperbilirubinemia.

2. BILIRUBIN METABOLISM

2.1. Bilirubin production

Bilirubin is produced by the degradation of heme (Figure 1A). The major source of heme (~80%) is hemoglobin, released after the breakdown of erythrocytes. The remaining 20% is derived from the catabolism of heme-containing proteins, such as myoglobin, peroxidase, cytochromes and catalase.[1,2] The life span of erythrocytes is approximately 120 days in adults, 70-90 days in neonates, and 50-60 days in rats.[3] Senescent erythrocytes are removed from the circulation and broken down in the reticuloendothelial system (RES), mainly localized in the liver, spleen, and bone marrow. The macrophages of the RES phagocytize heme and degrade it to bilirubin. Macrophages have two essential enzymes for heme degradation, namely heme oxygenase and biliverdin reductase.[4] Heme is first degraded by microsomal heme oxygenase, which results in the formation of equimolar quantities of iron, carbon monoxide (CO) and blue-green biliverdin IXα.[4] In most non-placental species biliverdin IXα is the end product of heme degradation.[5] In humans, however, biliverdin is subsequently converted by the cytosolic enzyme biliverdin reductase to the yellow-orange bilirubin IXα, also known as unconjugated bilirubin (UCB; Figure 1A).[6]

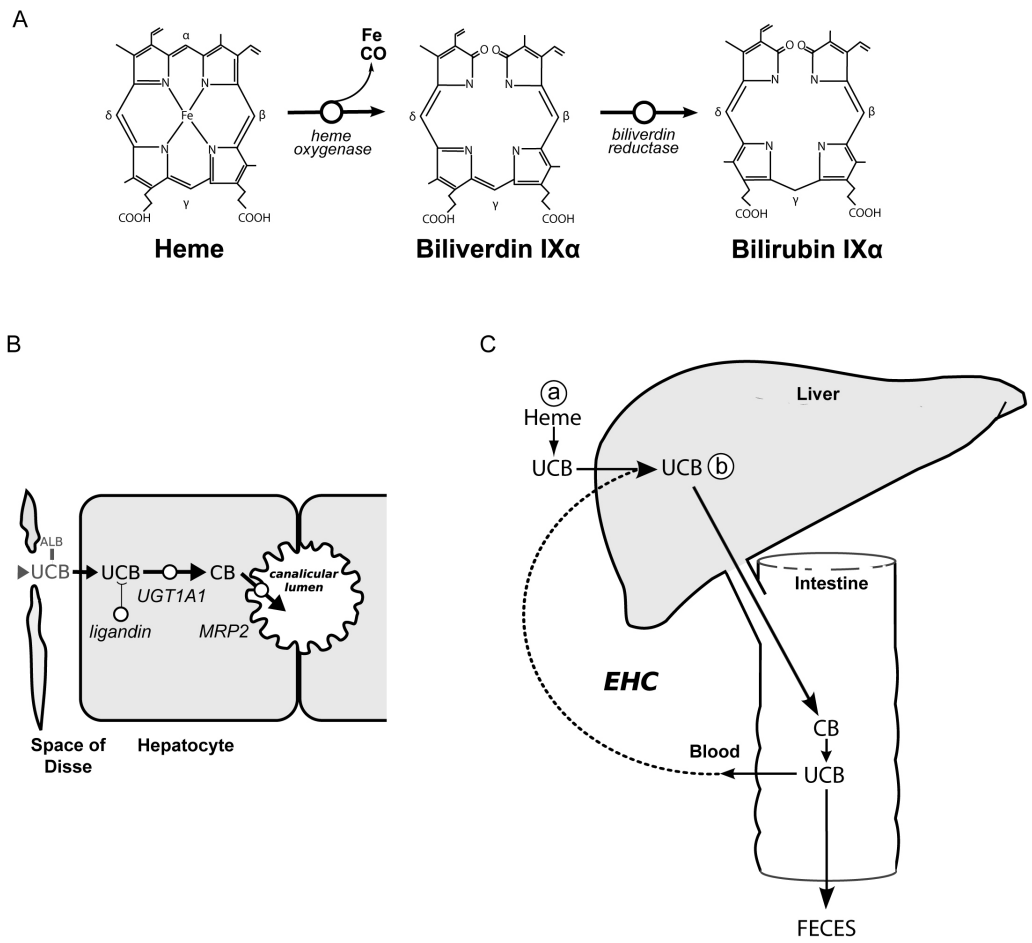

Figure 1. Bilirubin metabolism.
A. Bilirubin production in the macrophages of the reticuloendothelial system. **B.** Hepatic clearance of bilirubin. **C.** Enterohepatic circulation of bilirubin. (a) and (b) refer to previous panels.
CB: conjugated bilirubin, EHC: enterohepatic circulation.

Why the nontoxic, water-soluble biliverdin is converted to the potentially toxic and hydrophobic UCB is unclear. One possible explanation could be that in the fetus conversion of biliverdin to lipophilic UCB facilitates its excretion, since UCB can readily cross the placenta.[7] Secondly, UCB is a potent antioxidant, which may have beneficial effects in neonates.[8] The production rate of UCB is approximately 6-8 mg/kg per 24 hours in healthy full-term infants, and 3-4 mg/kg per 24 hours in healthy adults.[9,10] Infants have a higher production rate because of their higher red blood cell (RBC) count, the relatively larger fraction of hepatic heme proteins, and the shorter life span of fetal RBC's. Fetal hemoglobin (HbF), which has a higher affinity for oxygen than "adult"

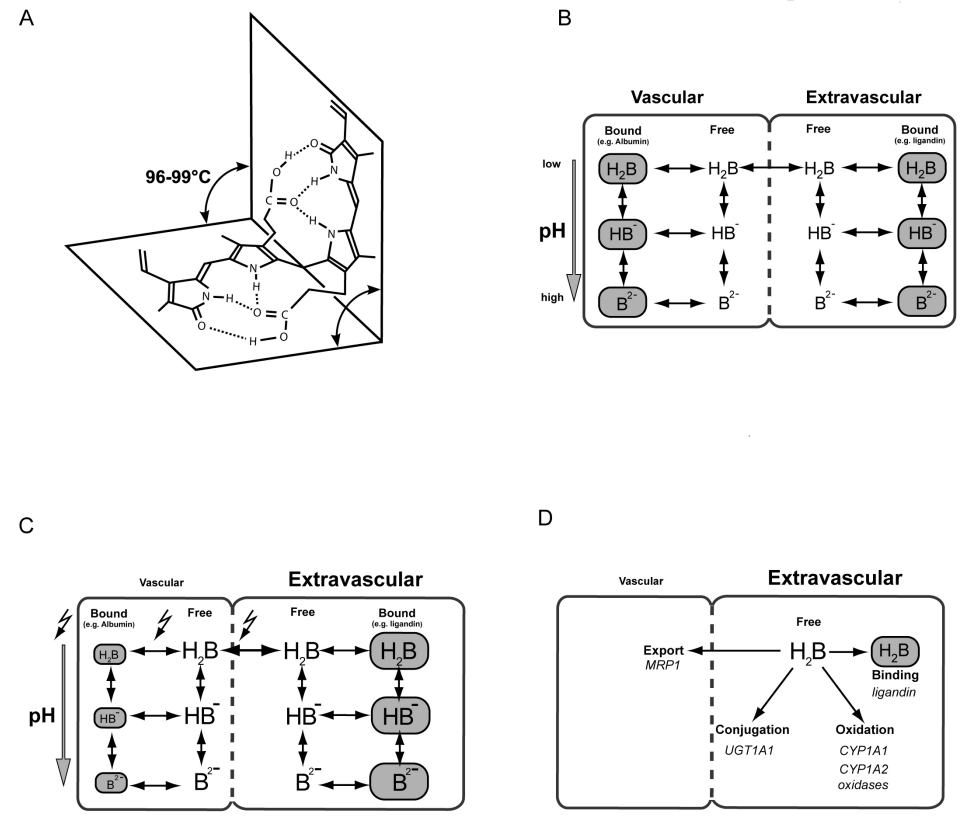

Figure 2. Bilirubin properties.
A. The three-dimensional structure of bilirubin. **B.** The three species of UCB (H_2B, HB^-, and B_2^-) can occur either bound or unbound throughout the body. The pH determines UCB ionization and thus the contribution of these three species to the total UCB pool. Only the free diacid (H_2B) diffuses from the plasma pool into the extravascular (tissue) pool and is consequently considered the toxic species of bilirubin. **C.** UCB can redistribute from the plasma to the tissue pool (*e.g.* the brain) if the amount of free H_2B increases in plasma. This may occur during hypoalbuminemia or in the presence of bilirubin displacing drugs. Additionally, factors that damage the blood-brain barrier and possibly factors that decrease the pH may further facilitate the entry and precipitation of free H_2B into the brain. **D.** Cells can decrease their free H_2B concentration *via* export, conjugation, oxidation, and binding.

HbA, is rapidly degraded postnatally in the relatively oxygen-rich environment of the pulmonary oxygenation. Conversion by heme oxygenase of one heme molecule to biliverdin produces one molecule of CO. Measurement of CO production, which is excreted *via* the lungs into expired air, can thus be used as an index of *in vivo* bilirubin production. UCB is released to the blood after its production in the RES. Once in the blood, the vast majority of UCB (99%) is reversibly bound to plasma albumin and in this way transported to the liver. Only 1% remains unbound, and is

accordingly called free bilirubin (B_f). The role of B_f in bilirubin-induced neurotoxicity is highly important, since only B_f, and not albumin-bound bilirubin, is able to translocate the blood-brain barrier and enter the brain (see paragraph 3.1 for more detail).[11,12] Each human albumin molecule has one high-affinity binding site for UCB. Beyond a molar ratio of 1:1, which roughly corresponds to an UCB concentration of 600 μmol/L, UCB can bind to additional sites on the albumin molecule with a lower affinity.[13,14] UCB is practically insoluble in plasma (less than 0.1 μmol/L) in the absence of albumin or apolipoprotein D.[15]

2.2. Structure and properties of bilirubin

The systematic name of UCB (Bilirubin IXα) is 1,8-dioxo-1,3,6,7-tetramethyl-2,8-divinylbiladiene-a,c-dipropionic acid.[4,5,16] UCB is a tetrapyrrole consisting of two rigid, planar dypyrroles that are joined by a methylene (-CH_2-) bridge at carbon atom 10.[17,18] The bilirubin molecule is preferentially shaped as a partially open book (Figure 2A). This confirmation is stabilized by 6 internal hydrogen bonds between the two dipyrroles, which can be looked upon as the covers of this book.[15,19] The resulting rigid confirmation renders bilirubin practically insoluble in water.[20] This is because the hydrophilic -COOH and -NH groups are placed in between the two dipyrroles, and are consequently shielded from interaction with water within the molecule. Conjugation, but also oxidation and interaction with light (*e.g.* phototherapy) opens the internal hydrogen bonds, which makes the UCB more hydrophilic and allows it to be readily excreted *via* the bile.[15,19,21]
Due to conjugation, bilirubin becomes a much better substrate for *e.g.* the canalicular multidrug resistance-related protein 2 (MRP2) transporter.
Depending of the pH of its environment, UCB can exist as either of three species with different degrees of ionization: diacid, monoacid, and dianion (Figure 2B).[15]
With each ionization a hydrogen bond is broken, which exposes the polar –COOH groups that can interact with water. In the plasma, at pH 7.4, more than 80% is present as diacid (H_2B), whereas ~18% is present as monoacid (HB^-), and less than 2% is present as the dianion (B^{2-}).[15] Each of the three species may exist either in a bound or in an unbound ("free") state (Figure 2B).[15] It is generally accepted that UCB bound to albumin, or other plasma proteins, is not toxic. The reason for this is the inability of the UCB-albumin complex to cross the endothelial layer in the absence of fenestrae (such as for example is the case in the central nervous system that is shielded by the blood-brain barrier).[12,22,23] Of the unbound species, free HB^- or B^{2-} are relatively harmless, since their polarity prevents them from diffusing across cell membranes.[14,24,25] The free diacid (H_2B) is considered to be the toxic form of UCB. The reason for this is that free H_2B, due to its hydrophobicity (<70 nmol/L in water)[20], is able to diffuse across cell membranes and enter the central nervous system (CNS) (Figure 2B).[12,25-30]

2.3. Hepatic clearance of bilirubin

Albumin transports UCB to the liver where it reaches the microcirculation of the sinusoids (Figure 1B). The sinusoidal endothelial layer has a fenestrated architecture that allows the albumin-UCB complex to enter the subendothelial space of Disse.[31] Once the complex is in direct contact with the hepatocyte membrane, UCB dissociates from albumin and is taken up as B_f by the hepatocyte. This transfer of UCB is most likely carrier-mediated, involving transporters that are not yet identified, although diffusional uptake of B_f has also been reported.[21,26,32-34] The B_f flux across the hepatocyte membrane can occur bi-directionally. To facilitate directional transport, B_f is bound in the cytosol to gluthathione S-transferase, traditionally referred to as ligandin or Y-protein, which decreases the intracellular B_f concentration and thus prevents UCB from re-entering the circulation (Figure 1B).[35-38]

2.4. Bilirubin conjugation

The next step in bilirubin catabolism involves conjugation of UCB. Conjugation is required since only conjugated water-soluble bilirubin, as opposed to non-polar, water-insoluble UCB, can be efficiently excreted *via* the bile. The enzyme bilirubin-uridine-diphosphoglucuronosyltransferase (UGT1A1), primarily located in the endoplasmic reticulum[39], catalyzes the transfer of one molecule of glucuronic acid from UDP-glucuronate to one of the carboxyl side chains of bilirubin, which produces bilirubin-monoglucuronide. Enzymatic addition of another glucuronic acid to the second carboxyl side chain of bilirubin, catalyzed by the same enzyme, produces bilirubin-diglucuronide, the fully conjugated form of bilirubin.[40] The conjugated form of bilirubin can be excreted with the bile, since it is more water soluble due to the adding of one or two polar glucuronic acid groups. Also, conjugation changes the three-dimensional structure of the bilirubin molecule and exposes, normally internalized, hydrophilic groups.[19,21] Absence of UGT1A1 in Crigler-Najjar disease results in unconjugated hyperbilirubinemia (see paragraph 4.3).

2.5. Bilirubin excretion

Under physiological conditions, a very small amount of UCB is excreted into bile without conjugation, where it rapidly associates with mixed micelles.[41] UCB in bile is seldom more than 2% of total bilirubin and is believed to originate in large part from hydrolysis of secreted conjugates in the biliary tree.
Conjugated bilirubin is excreted from the hepatocyte into the canalicular bile *via* the multidrug resistant protein 2 (MRP2, Abcc2).[42-44] The excretion of conjugated bilirubin is ATP-dependent and overcomes a steep concentration gradient (*i.e.* the bilirubin concentration in the bile is approximately 100-fold higher than in the hepatocyte).[44] In human bile ~80% of the conjugated bilirubin species appear as bilirubin diglucuronide, and ~20% as bilirubin monoglucuronide.[45] Again, this illustrates the importance of bilirubin conjugation, prior to its efficient excretion *via* the bile.

2.6. Enterohepatic circulation of bilirubin

Bilirubin transits the biliary tree and enters the intestinal lumen, where most of the conjugated bilirubin is hydrolyzed to UCB (Figure 1C). Hydrolysis can occur non-enzymatically under influence of mild alkaline conditions in the duodenum or jejunum.[46] Mostly, hydrolysis of conjugated bilirubin to UCB occurs enzymatically by β-glucuronidase.[21] This enzyme exists in the enteric mucosa and liver[47], but most of its activity in adults is of bacterial origin.[48,49] UCB becomes available for reabsorption after hydrolysis, a route which is not possible for conjugated bilirubin. Reabsorbed UCB is transported to the liver *via* the vena porta, where it is once again conjugated and excreted *via* the bile. This pathway is called the enterohepatic circulation (EHC) of bilirubin (Figure 1C).[50,51] In neonates, a high mucosal β-glucuronidase activity, and a relative lack of bacterial flora that metabolizes UCB into non-bilirubin metabolites, increases the EHC of UCB.

Conditions that increase the intestinal UCB concentration, such as fasting, seem to enhance the reabsorption and EHC of UCB. This leads to a proportional increase in systemic plasma UCB concentrations, due to a relatively low (~30%) first pass hepatic extraction of UCB from the vena porta. Conditions that increase the plasma UCB concentration are able to reverse the direction of B_f transport across the intestinal mucosa (*i.e.* from the blood into the intestinal lumen). During severe unconjugated hyperbilirubinemia, the direct excretion of B_f from the blood into the intestinal lumen indeed becomes a major route for bilirubin disposal from the body.[52,53] Taken together, the available data suggest that B_f transport across the intestinal mucosa occurs (at least predominantly) passively, and is driven by the concentration gradient between B_f in the plasma and B_f in the intestinal lumen. Although it has been shown that B_f is indeed capable of spontaneous diffusion across membranes[26], the occurrence of carrier-mediated UCB transport can not be excluded. Preventing EHC of UCB is a strategy for treatment of unconjugated hyperbilirubinemia.

Most of the UCB entering the intestinal lumen, however, is not reabsorbed, but metabolized by the intestinal microflora. Bacterial breakdown of UCB starts with the reduction of its tetrapyrrole ring, which converts UCB into urobilinogen. Urobilinogen can be oxidized to the yellow-orange urobilin. The brown color of feces is due to dipyrrolic oxidative derivates of UCB, the mesobilifuscins. Absence of urobilinogen in urine or feces indicates complete obstruction of the bile duct.

Urobilinogen can further be reduced to stercobilinogen. Urobilinogen and stercobilinogen are members of the urobilinoid family, *i.e.* the most important bacterial UCB breakdown products.[54] Several *Clostridiae* species (*C. ramosum, C. difficile*, and *C. perfrigens*) and *Bacteroides fragilis* are able to reduce UCB, but not conjugated bilirubin, to urobilinoids in the colon.[54-59] The efficacy of bacterial UCB degradation is illustrated by the observation that urobilinoids are the predominant bile pigment in stools from adult humans.[10,21] A small fraction of the intestinal urobilinoids are reabsorbed from the intestinal lumen and subsequently excreted *via* the liver or the kidneys.[21,54] Generally, almost all bilirubin is excreted *via* the feces. Human studies using ^{14}C-bilirubin showed that 92% of the daily bilirubin disposal occurs *via* the feces, and only 8% *via* renal excretion.[10,53]

2.7. Alternative metabolic routes of bilirubin disposal

When the disposal of UCB is impaired, as in patients with Crigler-Najjar disease (see paragraph 4.3), or in the animal model for the disease the Gunn rat (paragraph 5), UCB may be catabolized *via* alternative pathways. These include transmucosal diffusion and oxidation of bilirubin.

Transmucosal diffusion

UCB can, in its unbound form, freely diffuse across the intestinal mucosa.[14,50,51] Accumulation of UCB in the intestine will consequently induce a flux of B_f from the intestinal lumen into the blood.[60] The flux from gut to blood occurs in conditions that delay the intestinal transit, such as inadequate feeding or intestinal obstruction (see paragraph 4.4). The direction of the flux is inversed (*i.e.* from the blood into the intestinal lumen) if plasma UCB concentrations become excessively elevated. This occurs for example in patients with Crigler-Najjar disease, and in its animal model the Gunn rat (Figure 3).[51,61-63] In Gunn rats, it has been demonstrated that transmucosal diffusion is the most important alternative route for bilirubin excretion.[51,62]

Bilirubin oxidation

When conjugation of UCB is deficient, part of the UCB can be catabolized *via* the alternative pathway of oxidation. Oxidation of UCB leads to more polar metabolites that can be excreted into the bile. Hydroxylated products have been identified in the bile of Gunn rats.[64,65] Microsomal cytochrome P450 enzymes, such as Cyp1a1 and Cyp1a2, catalyze oxidation of UCB. In young Gunn rats, these enzymes are markedly upregulated.[66] The constitutively expressed non-inducible mitochondrial bilirubin oxidase, which can also oxidize bilirubin, has been demonstrated in the liver, intestine, and kidney of guinea pigs and rats.[67-69] Enzymatic oxidation of bilirubin has also been reported in brain, lung, heart and skeletal muscle.[67]

3. BILIRUBIN TOXICITY AND ANTIOXIDANT PROPERTIES

3.1. Molecular aspects of bilirubin-induced neurotoxicity

As stated above, B_f, *i.e.* bilirubin not bound to albumin, can cross the blood-brain barrier and enter the central nervous system (Figure 2C). During hypoalbuminemia, the fraction of B_f in the blood may increase. An increase in B_f can also be induced by substances that displace bilirubin from albumin (*e.g.* sulfonamides or free fatty acids).[70,71] When the blood-brain barrier is damaged, bilirubin can also enter the brain. Conditions that alter the blood-brain barrier include asphyxia, prematurity, infection, sepsis, hyperosmolarity, and hypercapnia.[72] Bilirubin accumulation in the CNS tissue is counteracted by the ATP-binding Cassette (ABC) protein MRP1.[30,73-75] This transporter is expressed in the epithelium of the choroid plexus and in the capillary endothelium of the brain. MRP1 actively pumps B_f out of the CNS. Furthermore, MRP1 is expressed in astrocytes and in neurons, and in this way they can regulate their intracellular B_f concentration (Figure 2D).[30,73-75]

MRP1 is not the only defense mechanism of neuronal cells against bilirubin-induced toxicity. Intracellular bilirubin can also be detoxified by oxidation, conjugation, and binding to cytosolic B-ligandin (Figure 2D).[25] When the interstitial B_f concentrations remain below aqueous saturation (70 nmol/L), these neuroprotective mechanisms prevent damage.[76] However, further increase in B_f concentrations may induce neurotoxicity at levels as low as 71-85 nmol/L.

The further increase in neurotoxicity is especially true at a low pH, which may decrease the solubility of bilirubin, and thus causes precipitation of insoluble H_2B aggregates. Exposure of neurons to B_f can decrease their excitability and synaptic transmission.[77] The decrease in excitability is due to a direct disruption of cell membrane integrity[78,79], and UCB-induced disturbances in calcium homeostasis and/or glutamate efflux.[79,80] B_f exposure also disrupts the integrity of mitochondrial and plasma membranes.[81,82] The following release of cytochrome C triggers apoptosis *via* the mitochondrial pathway.[83,84] Cytokines from microglial cells and astrocytes are released upon B_f exposure, which may further contribute to neurological damage.[85] Taken together these observations suggest that B_f disrupts several vital cellular functions. Importantly, *in vitro* studies showed that less differentiated (younger) nerve cells were more susceptible to bilirubin-induced neurotoxicity. The higher susceptibility is in concordance with the increased vulnerability of premature neonates to bilirubin-induced neurotoxicity.[85]

3.2. Clinical aspects of bilirubin-induced neurotoxicity

For yet unidentified reasons, bilirubin predominantly accumulates in the deep nuclei of the brain upon severe hyperbilirubinemia. The accumulation results in a yellow staining of the basal ganglia, the hippocampus, various brain stem nuclei (*e.g.* oculomotor, cochlear, vestibular and olivary nuclei), and the cerebellum.[86] The yellow staining is also known as kernicterus. Kernicterus was described for the first time as an autopsy finding in neonates that had died during severe unconjugated hyperbilirubinemia.[87] Originally kernicterus was a pathological diagnosis. Nowadays, the term is also used to describe the acute and chronic neurological syndrome. The acute form of kernicterus consists of three phases.[72,86] The first phase is characterized by poor sucking, hypotonia, stupor, and seizures. In the second phase, the infant can develop a hypertonia of the extensor muscles, which can be accompanied by opisthotonus, retrocollis, fever and a high-pitched cry. During the third phase, which generally starts after the first week, the hypertonia is gradually replaced by hypotonia. Currently, in stead of "acute kernicterus" the term "acute bilirubin encephalopathy" is more often used.[12] We also know a chronic form of kernicterus. The chronic form is basically characterized by hypotonia in the first year, and movement disorders (*e.g.* choreoathetosis, ballismus, and tremor), dental dysplasia, vertical gaze paralysis, and sensorineural hearing loss thereafter. A more subtle clinical picture, which involves hearing loss and a mildly impaired neurologic and cognitive performance, is referred to as bilirubin-induced neurological dysfunction (BIND).[86] At the moment, the risk-assessment to develop kernicterus, and guide treatments to prevent this, are based on total plasma bilirubin concentrations in

jaundiced neonates and in patients with Crigler-Najjar disease. However, studies in animals and neonates have shown that plasma B_f concentrations are more closely related to the development of bilirubin-induced neurotoxicity.[12,28,71,88,89] Therefore, incorporation of plasma B_f concentrations in the clinical evaluation of unconjugated hyperbilirubinemia in neonates and patients with Crigler-Najjar disease would be recommendable. Currently, however, measuring B_f is not routinely incorporated in clinical practice. The main reason for this lies in the inaccuracy of the commercial B_f test, most notably caused by a 42-fold sample dilution that alters bilirubin-albumin binding.[90]

3.3. Bilirubin-induced toxicity in other organs

Apart from the brain, UCB may also have harmful effects on other organs.

Gunn rats develop kidney damage due to the deposition of bilirubin crystals and necrosis, which lead to an impaired concentration capacity of urine by the kidneys and polyuria.[91,92] Reduced kidney function has also been observed in jaundiced neonates.[93] Bilirubin deposition in the teeth may cause dental enamel dysplasia or green discoloration of the teeth.[94] Patterns of bilirubin deposition have also been found in heart, lung, adrenal, pancreas, testis and skin.[95,96] The liver is relatively resistant to bilirubin-induced toxicity, which might be due to its high degree of protein binding, and its ability to conjugate bilirubin.[97]

3.4. Bilirubin as an antioxidant

As described above, heme oxygenase and biliverdin reductase catalyze the conversion of heme into UCB. The enzyme that catalyzes the first step, heme oxygenase, has an important anti-oxidative role in both humans and animals. The important anti-oxidative role might be due to its ability to convert pro-oxidative heme into biliverdin, a known anti-oxidant. The carbon monoxide that is released during this conversion has several beneficial effects as anti-proliferative, anti-inflammatory, as well as vasodilatory properties. Remarkably, mammals subsequently convert the fairly harmless biliverdin into the more toxic bilirubin. Luckily, however, UCB also appears to be a potent anti-oxidant. In the 1950s UCB was already reported to protect linoleic acid and vitamin A from oxidation.[98] Subsequent studies showed that UCB scavenges singlet oxygen and peroxyl radicals[8,99], and might prevent membrane lipid oxidation.[99] In the 1980s it became clear that the antioxidant capacity of UCB exceeds that of vitamin E towards lipid peroxidation.[8,100] These observations were later supported by more clinical studies in both neonates and adults.[101] Neonates with illnesses (*e.g.* sepsis, asphyxia) believed to enhance free radical production, had a significantly lower daily rise in mean plasma bilirubin concentrations than control infants.[102,103] The lower daily rise in bilirubin is consistent with the hypothesis that bilirubin is consumed as an antioxidant. In adults, many studies have established an opposite relationship between plasma bilirubin concentrations and cardiovascular diseases (see for review:[101]). This anti-atherogenic effect might be due to the fact that UCB inhibits the oxidation of low density lipoproteins.[104,105] Furthermore, elevated bilirubin levels were associated with a decreased risk of cancer mortality.[106] Based on animal studies, bilirubin might also protect from ischemia-reperfusion injury.[107,108]

4. UNCONJUGATED HYPERBILIRUBINEMIA

4.1. Introduction

Hyperbilirubinemia can originate from different causes, but eventually results from an imbalance between bilirubin production and bilirubin excretion.[109] Hyperbilirubinemia can either be unconjugated or conjugated, or involves both UCB and conjugated bilirubins. Conjugated hyperbilirubinemia, *i.e.* the accumulation of bilirubin conjugates, always involves a pathophysiological mechanism located after the level of hepatic conjugation. The defects can be localized within the hepatocyte (*e.g.* inactive MRP2 in Dubin-Johnson syndrome, or viral hepatitis) or within the biliary tree (*e.g.* obstructive jaundice due to malignancies or bile stones). This introductory chapter will be limited to unconjugated hyperbilirubinemia, which can result from increased UCB production, decreased hepatic clearance, or increased enterohepatic circulation of UCB.

Unconjugated hyperbilirubinemia becomes clinically apparent in the form of visible jaundice at plasma bilirubin concentrations at or above 85 μmol/L.[110] Normal plasma total bilirubin levels in human adults range from 5 to 17 μmol/L. Up to 70% of term neonates and almost all preterm neonates develop a transient elevation in plasma UCB concentrations within the first week of life. The transient elevation of UCB is due to a combination of an elevated erythrocyte turnover (increased production), immature hepatic conjugation of bilirubin (decreased hepatic clearance) and a delayed intestinal transit (enhanced enterohepatic circulation of UCB; Figure 1).[72] For the majority of neonates this results in a benign transitional phenomenon, so-called physiological jaundice, which may be beneficial, due to the antioxidant properties of UCB.[101,111] However, in some cases a pathological unconjugated hyperbilirubinemia develops. Jaundice is considered pathological if the levels of UCB increase too fast (*i.e.* jaundice within the first postnatal day, or a rise in serum UCB concentration that exceeds 3.4 μmol/L per hour), rises too high (*i.e.* exceeding the 95th percentile for age in hours), or remains elevated for more than 2 weeks (icterus prolongatus). Pathologic unconjugated hyperbilirubinemia is generally due to an exaggeration of the mechanisms that caused physiological jaundice, *i.e.* increased bilirubin production, decreased hepatic clearance, and/or an enhanced enterohepatic circulation of bilirubin.[72] These causes for unconjugated hyperbilirubinemia, as well as the resulting clinical conditions, will be discussed in more detail below.

4.2. Increased bilirubin production

Neonates produce 7-11 mg UCB per kg per day, while adults produce about one-third of this amount.[9,10] This relatively high UCB production is due to the combination of a high erythrocyte count, a short erythrocyte life-span (70-90 days), and an enhanced catabolism of fetal hemoglobin (HbF) in neonates compared with adults. Increased UCB production is a major contributor to

physiologic jaundice.[112] Excessive UCB production can cause pathological jaundice and is frequently observed during neonatal hemolytic disease.

Hemolytic disease

Hemolysis increases bilirubin production. Hemolysis can be iso-immune mediated (*e.g.* blood group ABO or Rhesus (D) incompatibility), related to inherited cell membrane defects (*e.g.* hereditary spherocytosis), to erythrocyte enzymatic effects (*e.g.* glucose-6-phosphate dehydrogenase deficiency), or to sepsis.[72] Any of these conditions may result in a severe, transient unconjugated hyperbilirubinemia in neonates, partly because of the immature liver. Extravasation of blood (*e.g.* cephalhematoma, intracranial hemorrhage), may also contribute to a high UCB load in neonates.[72] In adults, hemolysis does not induce such an excessive unconjugated hyperbilirubinemia, because of the generally efficient conjugation and clearance of bilirubin by the mature liver.

4.3. Decreased hepatic clearance of bilirubin

In neonatal hepatocytes, B-ligandin levels are relatively low (Figure 1B).[113,114] However, the decreased hepatic uptake of B_f has no major impact on the development of physiological jaundice.[46] On the other hand, the immature activity of the hepatic enzyme UGT1A1 is considered to be the rate-limiting step in neonatal bilirubin catabolism. It has been estimated in adults that the total capacity of UGT1A1 is about a 100-fold greater than needed to clear the B_f load.[115] However, directly after birth the UGT1A1 activity is usually less than 0.1% of adult values, whereas on the other hand the substrate (UCB) supply is increased.[115] This activity increases exponentially, until adult levels are reached at 6 to 12 weeks of life.[116] Thus, the physiological decrease in UGT1A1 activity contributes to a great extent to the pathogenesis of physiological jaundice. The ability of all newborns to conjugate bilirubin is significantly impaired in the first few days, so even a small increase in the rate of UCB production can contribute to the development of hyperbilirubinemia. The impaired conjugation suggests, that an increased heme catabolism might be even a more important mechanism responsible for hyperbilirubinemia than the decreased UGT1A1 activity, in the first days after birth.[112] The two most important inherited defects in bilirubin conjugation are Gilbert's syndrome and Crigler-Najjar disease. These diseases will be discussed in more detail below.

Gilbert's syndrome

Gilbert's syndrome is an inherited defect in bilirubin conjugation that occurs in 7-12% of the population.[117,118] It was first described by Gilbert in 1901[119], and is a mild recurrent unconjugated hyperbilirubinemia that usually does not become manifest until after the second decade of life. The mode of inheritance is considered autosomal recessive, although heterozygote carriers do have slightly elevated levels of plasma UCB.[120,121] The disease is characterized by a 70-80% decrease in

UGT1A1 activity, which is due to a polymorphism (an extra TA in the TATAA box) in the promoter region of the UGT1A1 gene.[120,122] Since this decrease in activity does not exceed the reserve capacity of UGT1A1, it will normally not result in jaundice. Concomitant hemolysis, however, may result in a build-up in plasma UCB that exceeds 85 μmol/L, which is the threshold for visible jaundice. Concomitant hemolysis occurs for example in newborns with Gilbert's syndrome or with glucose-6-phosphate dehydrogenase deficiency[61], or in adults in conditions that increase the UCB load, such as fasting and intercurrent illness.[123] Therapy is not required for Gilbert's syndrome patients, and the most important aspect of care involves recognition of the disorder. It is useful to explain to a patient under which conditions jaundice may occur, and that Gilbert's syndrome has an inconsequential nature. Interestingly, there are indications that patients with Gilbert's syndrome are protected from developing ischemic heart disease, which would concur with the beneficial antioxidant capacity of bilirubin.[124]

Crigler-Najjar disease

Crigler-Najjar disease is a rare autosomal recessive inherited disease characterized by permanent unconjugated hyperbilirubinemia since birth. The prevalence is estimated at 1:1,000,000.[125] In the Netherlands there are approximately 25 patients. The disease has been classified into two distinct forms.

Crigler-Najjar disease type I was first described in 1952 and is caused by a complete absence of UGT1A1 activity.[126] It is caused by mutations (including deletions, insertions, and premature stop codons) within the five exons of the UGT1A1 gene that lead to an inactive form of the enzyme.[122,127] Untreated, plasma UCB concentrations would range between 350-800 μmol/L and patients would develop kernicterus and die.

Crigler-Najjar disease type II was defined in 1962 by Arias.[128] In type II, the genetic lesions are also located within the 5 exons of the gene, but consist exclusively of point mutations. The genetic lesions result in an activity of the UGT1A1-enzyme of less than 5% of normal.[127]

Patients with Crigler-Najjar disease type I suffer from a life-long, severe unconjugated hyperbilirubinemia.[126] These patients rely on daily phototherapy (up to 16 hours a day) to prevent kernicterus. The strict treatment regime has a great impact on social life, which tends to decrease compliance over time.[129] Furthermore, the efficiency of phototherapy also decreases with age, due to skin thickening, increased pigmentation, and a decrease in the surface area to body mass ratio.[125,129-131] Before the introduction of phototherapy, all patients with Crigler-Najjar disease died from kernicterus.[126] Exacerbations of jaundice due to fasting or concurrent illness may be treated with intensive phototherapy, albumin administration, exchange transfusion, and oral amorphous calcium phosphate in order to prevent brain damage.[125,129-131] Despite this intensive treatment, approximately one-fourth to one-half of the patients with Crigler-Najjar disease type I develop mild to severe brain damage. In addition, 9-38% die resulting from complications related to the disease.[129-133] Eventually, liver transplantation is presently the only definitive treatment for Crigler-

Najjar disease type I. Despite the risks in terms of morbidity and mortality, some transplant centers advocated that liver transplantation should be performed at a young age to prevent irreversible brain damage.[129] In the Netherlands it has remained standard practice to postpone transplantation till proven therapeutic inadequacy of other treatment modalities.

Patients with Crigler-Najjar disease type II (*i.e.* Arias syndrome) still have residual UGT1A1 activity, which results in a milder phenotype.[128] Importantly, these patients are to some extent still able to conjugate and excrete bilirubin *via* the bile.[134] Bile of type I patients contains virtually no conjugated bilirubin, whereas bile of type II patients contains predominately mono-conjugates and some bi-conjugates.[134] Daily phototherapy is generally not required in type II patients, since the plasma UCB concentration usually remains below 350 μmol/L. If necessary, type II patients can be treated with phenobarbital, which enhances the residual UGT1A1 activity and decreases plasma UCB concentrations by approximately 30%.[134,135]

4.4. Increased enterohepatic circulation of bilirubin

In neonates, the high mucosal β-glucuronidase activity leads to an almost complete hydrolysis of conjugated bilirubin in the intestinal lumen.[50,51] After hydrolysis UCB can either be degraded by the intestinal microflora, reabsorbed into the EHC, or excreted *via* the feces without further metabolism (Figure 1C).[50,51] However, the anaerobic microflora which is needed for this degradation, does not develop until 2-6 weeks after birth.[136] As a result, almost all intestinal UCB becomes available for reabsorption into the EHC.[137] Given that the liver extracts only 30% of the UCB load, in a first pass effect, this increased EHC will directly contribute to the development of physiological jaundice.[21] The high β-glucuronidase activity and the relative lack of intestinal microflora thus contribute to the development of physiological jaundice.[136] Inadequate feeding, breast-milk jaundice, and an anatomic or functional intestinal obstruction may further increase plasma UCB levels to pathological levels and will be discussed in more detail.

Inadequate feeding

Inadequate feeding often results from insufficiently effective breastfeeding. Ineffective breastfeeding may be due to a variety of maternal and neonatal factors, such as improper technique, engorgement and/or ineffective sucking of the neonate. When inadequate feeding results in a decreased intake, the neonatal weight loss may become increased and the physiological unconjugated hyperbilirubinemia in the first 3-5 days of life can become exaggerated.[60,138,139] In animal studies, starvation decreased fecal UCB excretion, increased UCB concentration in the intestinal lumen, and thereby increased the reabsorption of UCB into the EHC.[140,141] It is therefore likely that inadequate feeding exaggerates unconjugated hyperbilirubinemia in neonates *via* a similar mechanism (*i.e.* increased EHC of UCB). Indeed, early and frequent feedings in the first postnatal days revealed to decrease plasma UCB concentrations in neonates.[104,142]

Breast milk jaundice

Breast milk jaundice, which must be distinguished from jaundice due to inadequate feeding, typically begins after the first 3-5 postnatal days and peaks within two weeks after birth. It has been hypothesized that β-glucuronidase, present in human milk but not in infant formula, is responsible for the exaggeration of unconjugated hyperbilirubinemia, because it would enhance intestinal bilirubin hydrolysis.[143] However, this remains highly questionable since the amount of β-glucuronidase in human milk is relatively small compared with the quantities of the enzyme in the intestinal mucosa. Another possible explanation for breast milk jaundice relates to the highly efficient fat absorption from breast milk, in which case no carrier exist for intestinal UCB to leave the intestine *via* the feces.[144]

Intestinal obstruction

Intestinal obstruction (*e.g.* functional or anatomical) delays the intestinal transit. This leads to the accumulation of UCB in the intestinal lumen, and an increase in the EHC of bilirubin. Obstruction due to a delayed passage of meconium[145], pyloric stenosis[146], or Hirschprung's disease[147] are all associated with increased plasma UCB concentrations. Acceleration of the gastrointestinal transit, by rectal stimulation[148], frequent feedings[104,142], or feeding infant formula that contain oligosaccharides, reduces unconjugated hyperbilirubinemia in neonates.[149,150]

5. THE GUNN RAT MODEL

Many studies on pathophysiology or novel treatments of hyperbilirubinemia cannot (or at least, not easily) be performed in humans. In this thesis we used the Gunn rat as a model for unconjugated hyperbilirubinemia. In 1938, Gunn first described a spontaneously jaundiced mutant strain of Wistar rats, which have a life-long, severe unconjugated hyperbilirubinemia.[151] It was not until 1957 that the Gunn rat was recognized as an animal model for Crigler-Najjar disease type I, when it was demonstrated that the unconjugated hyperbilirubinemia in these animals was caused by an inherited inability to form bilirubin conjugates (Figure 3).[152,153] The Gunn rat also appeared to be a good model for bilirubin-encephalopathy in humans, especially when the amount of free bilirubin was further increased by concomitant hemolysis or by administration of albumin displacing drugs.[154-157] The neurologic lesions in Gunn rats resemble those in humans and include cell loss and gliosis in the auditory nuclei of the brainstem, the oculomotor nuclei, cerebellum, hippocampus and basal ganglia.[152,158-160] However, differences between Gunn rats and jaundiced humans do apply: for example, cerebellar hypoplasia is only a prominent feature of bilirubin-induced neurological damage in Gunn rats.[161] Changes in learning behavior, neurological function, and electrophysiology of the auditory nervous system have been observed both in Gunn rats and in humans.[62,158] Renal damage has also been described in both species.[91-93] The phenotype of Gunn rats is caused by a single frame shift mutation in the UGT1A1 gene, which leads to the formation of a truncated and

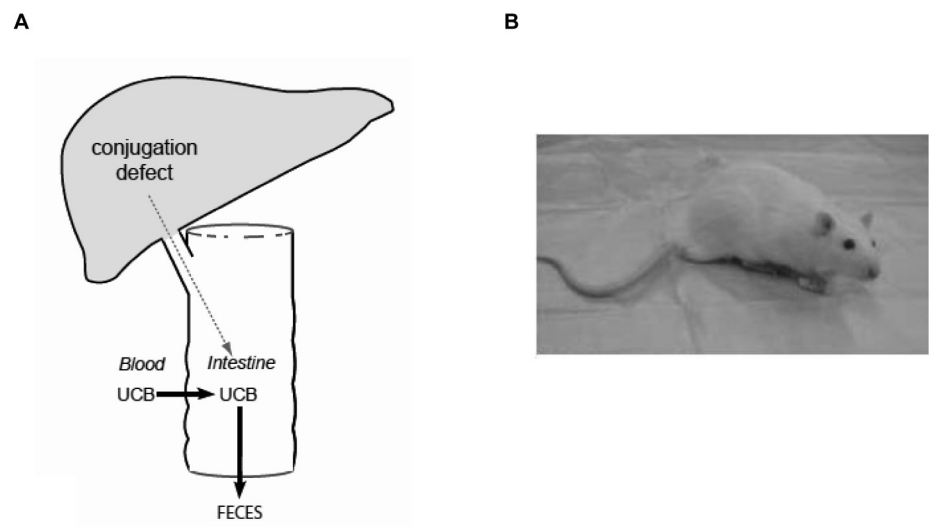

Figure 3. Unconjugated hyperbilirubinemia.
A. Patients with Crigler-Najjar disease type I, and their animal model the Gunn rat, are unable to conjugate bilirubin in the liver due to absent UGT1A1 activity. The resulting severe unconjugated hyperbilirubinemia results in diffusion of free UCB from the blood, across the intestinal mucosa, into the intestinal lumen. This transmucosal UCB diffusion is considered a major excretory pathway for UCB during severe unconjugated hyperbilirubinemia. **B.** The Gunn rat.

inactive enzyme.[152,153] Thus, the Gunn rat is a phenotypic and genotypic animal model for Crigler-Najjar disease type I. It is the most well-established animal model to study severe unconjugated hyperbilirubinemia and bilirubin-induced neurological damage.

Mouse model

Recently, Bortolussi *et al.* generated a novel mouse model for Crigler-Najjar disease type I by targeting a nonsense point mutation.[162] The mutation, a 1-base deletion in Ugt1 exon 4, is identical to that present in Gunn rats[163] and similar to many of those found in Crigler-Najjar type I patients.[164,165] This mouse model resembles most major features of the human syndrome, such as neonatal hyperbilirubinemia and early lethality due to bilirubin-induced neurological damage. The cerebellar architecture in the mouse is significantly affected, together with reductions in Purkinje cell number and dendritic arborization. As in untreated patients with Crigler-Najjar disease type I, the lack of UGT1A1 activity invariably results in death from bilirubin neurotoxicity.[126] In one study, neonatal lethality in newborn mouse pups was prevented by a single dose of adeno-associated viral vector 9 expressing the human UGT1A1. Gene therapy treatment completely rescued the mice, accompanied by lower plasma bilirubin levels and normal brain histology and motor coordination.[162] This novel mouse model represents a useful model to develop novel technologies to find a treatment option based on gene correction.

6. DIAGNOSTIC TOOLS FOR UNCONJUGATED HYPERBILIRUBINEMIA

Diagnosis of unconjugated hyperbilirubinemia is usually based on routine laboratory parameters, *i.e.* total serum bilirubin measurements. In addition, transcutaneous bilirubin measurements are increasingly being used to screen newborn infants for hyperbilirubinemia. Furthermore, auditory brainstem responses (ABRs) are a very sensitive parameter to detect imminent bilirubin neurotoxicity.

6.1. Total serum bilirubin

To prevent severe unconjugated hyperbilirubinemia and bilirubin toxicity, (inter)national guidelines are used to standardize management of jaundiced neonates.[166,167] Current management guidelines are based on the total serum bilirubin (TSB) concentrations. Measurement of albumin concentration is recommended because a low albumin concentration is considered a risk factor for bilirubin neurotoxicity, resulting in lower TSB treatment thresholds or albumin administration.[166] Recently, unacceptably high (interlaboratory) variability has been demonstrated in bilirubin and albumin measurements in the Netherlands.[168] The findings in the Netherlands are in agreement with data of the United States of America (USA), where similar interlaboratory variability of bilirubin measurements was reported.[169] Interlaboratory variability can be traced, at least in part, to height of the bilirubin concentration and to the use of different matrices (*i.e.* bovine versus human serum based), as has been addressed previously.[169-173]

To comply with international guidelines for the management of neonates with unconjugated hyperbilirubinemia in order to standardize treatment, exchangeability of bilirubin and albumin measurements among laboratories is essential. To analyze and improve the interlaboratory variability, a tailor made Quality Assessment Scheme for neonatal samples (human serum based) has become available in the USA since 2003[171,172] and in the Netherlands since January 2010.[168]

6.2. Transcutaneous bilirubin measurement

As stated above, treatment thresholds for phototherapy and exchange transfusion are momentarily based on TSB concentrations.[166,167,174] As a logical consequence, invasive blood sampling is considered necessary to measure TSB levels in the diagnosis and follow-up of treatment during hyperbilirubinemia. The measurement of TSB includes painful blood punctures, the risk of infection and, especially in preterm infants, can lead to significant blood loss. A non-invasive alternative method to screen for hyperbilirubinemia is transcutaneous measurement of bilirubin (TcB). Transcutaneous bilirubin values give an estimate of the TSB concentration based on the spectrophotometric measurement of the yellow color of the skin and subcutaneous tissue.[175,176]

The principle by which TcB meters work involves directing light into the skin of the neonate, and measuring the intensity of specific wavelength that is returned. The device analyzes the spectrum of optical signals reflected from the neonate's subcutaneous tissues. These optical signals are

converted to electrical signals by a photocell. The electrical signals are analyzed by a microprocessor to generate a serum bilirubin value. Different bilirubinometers exist, with different precision. In our hospital we use the Minolta Airshield (JM-103). Several recommendations on the use of TcB measurements exist, as shown in Table 1.

The TcB meter can be placed on the forehead or sternum of the neonate for the measurement.[177] TcB measurement can not be used during or after phototherapy. Through its bleaching effect on the skin, phototherapy affects the correlation between TcB and the bilirubin values.[178]

TcB measurements using the JM-103 instrument tend to underestimate TSB concentration by ~50 µmol/L.[179] Based on the underestimation of ~50 µmol/L we recommend for the management of hyperbilirubinemia in preterm infants to use a TcB plus 50 µmol/L cut-off level at 70% of the TSB phototherapy threshold. The use of this cut-off level is associated with a substantial reduction in the need for blood samples and a minimal risk to miss high TSB levels.[179] When the TcB plus 50 µmol/L exceeds the value where, based on the guidelines, phototherapy is needed, TSB levels should be determined. Also, in case of doubt a TSB value should be measured. TSB remains the "gold standard" in the management of hyperbilirubinemia.[177]

Table 1. Recommendations for the use of JM-103.

Recommendations for transcutaneous bilirubin measurements in preterm infants with and without phototherapy
• Measure TcB levels under the diaper on the hipbone of the infant.
• TcB cut-off level: Add 50 µmol/L to the measured TcB level at 70% of the PT threshold.
• Ensure regular calibration of the TcB device according to the recommendations of the manufacturer.
• Regularly perform paired TSB and TcB levels to check the accuracy of the TcB device.
• Teach NICU nurses and attending physicians how to perform and interpret TcB measurements.
• Measure the TSB level:
• If the TcB level plus 50 µmol/L exceeds 70% of the TSB PT threshold.
• If there is clinical concern on severe hyperbilirubinemia.
• If you do not trust the result of that specific transcutaneous measurement.

TSB: total serum bilirubin, TcB: transcutaneous bilirubin, PT: phototherapy, NICU: neonatal intensive care unit, 17.1 µmol/L = 1 mg/dL bilirubin.

6.3. Brainstem Auditory Evoked Potentials

Because exact neurotoxic TSB concentrations are unknown, and risk factors for imminent bilirubin neurotoxicity are not evidence based, another diagnostic tool, in addition to TSB, may be valuable to detect imminent BIND in jaundiced newborn infants.

The neural auditory pathway is very susceptible to bilirubin toxicity, putatively resulting in sensorineural hearing loss or auditory neuropathy, also known as auditory dys-synchrony. A frequently used, non-invasive and sensitive tool to determine bilirubin neurotoxicity is the Auditory

Brainstem Response (ABR), which allows for determination of electrophysiological activity of the neural auditory pathway. The ABR consists of a sequence of positive waves (numbered I-V) representing the electrophysiological conductance through the auditory pathway from inner ear to brainstem. Wave I and II represent the peripheral auditory nerve and waves III-V represent the activity in the auditory centers at the brainstem level of the pathway (cochlear nucleus and lateral lemniscus, respectively).[180] Bilirubin-induced ABR changes mainly involve waves III and V, and may progress from increased interwave latencies to the loss of wave amplitude. ABR changes can be transient, but may also progress into permanent wave changes or even loss of any recognizable wave.[156,181] A bedside method to evaluate the electrophysiological integrity of the auditory pathway is the Automated Auditory Brainstem Response (AABR), such as applied in ALGO hearing screening systems (Natus Medical, San Carlos, CA, USA). AABR assessments are simplified ABR measurements that are able to identify infants with abnormal cochlear or auditory function. A "pass" or "refer" result is shown on the ALGO machine for each ear of the infant.[182] In an observational study of 191 hyperbilirubinemic patients with variable birth weight (406-4727 g) and variable gestational age (24-42 weeks), an abnormal ALGO result (bi- or unilateral refer) was associated with increased B_f concentrations and B_f/TSB ratios, but not with TSB concentrations alone.[28]

In the Netherlands every healthy newborn gets a hearing screening between postnatal day four and seven. The screening is based on three tests with a step-up method. The first and second test are based on Oto-Acoustic Emissision (OAE). Only if the first and second test are abnormal the third test will be done, which is the ABR. In addition, all Dutch Neonatal Intensive Care Units (NICU) participate in a two-stage AABR neonatal hearing program, because infants admitted at a NICU have an increased risk of hearing loss.[183,184] The instruments' algorithm of the AABR assumes an infant as passing the test when the acquired data obtained at 35 dB fits with 99.96% likelihood a template composed of ABR from normal hearing newborns. Infants who fail the two AABR stages are referred to an audiological center for further diagnostics, including conventional ABR. All infants who are admitted longer than 24 hours are screened with ABRs to monitor hearing problems. This screening is done one day prior to discharge, and the infant should preferably be ~30 weeks (postconceptional age).[185]

7. TREATMENT FOR UNCONJUGATED HYPERBILIRUBINEMIA

7.1. Treatment options for Crigler-Najjar patients and neonates

Phototherapy

In 1956 a nurse in England noticed that jaundiced infants exposed to sunlight became less yellow.[186] Subsequently, pediatric resident Cremer elaborated on this observation and he demonstrated the efficacy of phototherapy by exposing preterm infants to blue fluorescent lights, which decreased

plasma bilirubin concentrations.[186] Since the mid 1960s phototherapy has extensively been used for treatment of unconjugated hyperbilirubinemia.

The peak wavelength of light absorption by bilirubin is in the blue region of the spectrum, near 460 nm.[187-189] Narrow spectrum blue lights (460-490 nm) are considered to be most effective for the treatment of unconjugated hyperbilirubinemia.[187,190] When bilirubin molecules in the skin absorb (phototherapy) light of this wavelength range, three photochemical reactions can occur: configurational photo-isomerization, structural photo-isomerization, and photo-oxidation (Figure 4). In configurational photo-isomerization, one (or both) of the double bonds at carbon atoms C4 and/or C15 in the bilirubin molecule is (are) opened. The configurational photo-isomerization changes it from the ZZ configuration to a ZE, EZ or EE (Z for *zusammen*, and E for *entgegen*).[191] When this occurs, the polar N and O groups are exposed, making the UCB-photoisomer less hydrophobic than UCB, and in this way a better substrate for transport into the bile *via* MRP2.[188,189] In structural photoisomerization, intramolecular cyclization of bilirubin occurs to form lumirubin.[192] Lumirubin is cleared much more rapidly from plasma than the isomer formed by configurational photoisomerization. Lumirubin is considered mainly responsible for the decrease in plasma UCB concentrations during phototherapy.[193,194] Finally, photo-oxidation hydrolyzes UCB into mono- and dipyrroles that can be excreted *via* the urine.[195] Photo-oxidation seems to play a minor role in UCB catabolism.

The efficacy of phototherapy in reducing TSB concentrations depends on several factors: the spectrum of the light emitted, the spectral irradiance of the light (energy output), the distance of the light source to the infant, the duration of light exposure, as well as on the skin area that is exposed to light.[196] All these factors should be routinely evaluated.[190] However, considering the position of the infant under phototherapy, it is known that the duration of the phototherapy is not decreased when preterm infants are turned every two hours from the suspine position to their back and *vice versa*, compared to infants who where not turned at all.[197] Also, it has been shown that intermittent phototherapy was as effective as continuous phototherapy.[198,199] The explanation for this observation was that the blanched skin would be reloaded with bilirubin when phototherapy is stopped periodically during intermittent phototherapy, thus increasing the efficacy of the phototherapy.

To ensure that the most optimal treatment is delivered to the infants, regardless of the design of the phototherapy unit, the Committee on Fetus and Newborn of the American Academy of Pediatrics (AAP) has made recommendations for phototherapy treatment.[200] The AAP defined optimal phototherapy as blue light in the emission spectrum of 460 to 490 nm delivered at a light irradiance of ≥30 μW/cm^2/nm to the largest possible body surface. For fluorescent tubes a few studies showed a dose-response relationship between light irradiance and decrease of TSB.[201-204] In one of the studies a linear relationship was found by using relatively low light irradiance.[201] Contrary to this, Tan *et al.* have suggested a "saturation point" of 30 μW/cm^2/nm, above which no further decrease in TSB is seen with increasing irradiance.[204] For light-emitting diodes (LEDs)

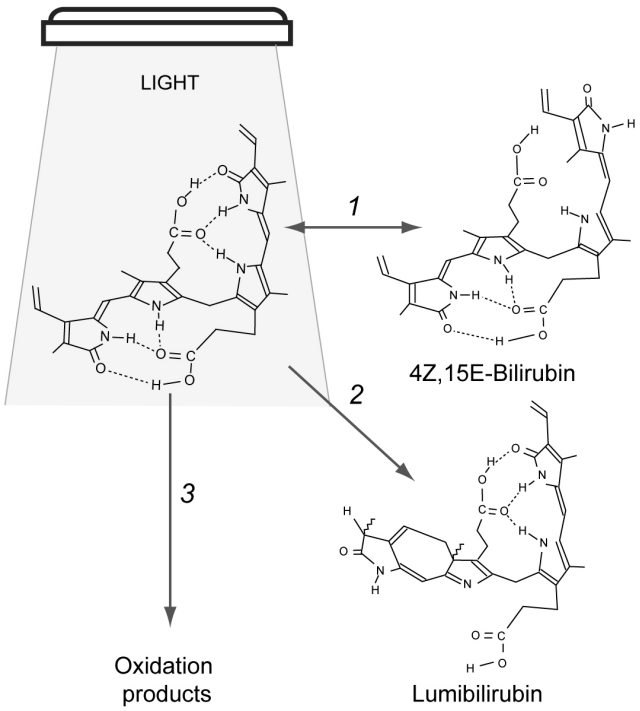

Figure 4. Treatment for unconjugated hyperbilirubinemia.
Phototherapy changes the configuration of (sub)dermal UCB *via* three distinct photochemical reactions: (1) configurational photo-isomerization, (2) structural photo-isomerization, and (3) photo-oxidation. The changes in configuration expose the hydrophilic groups of bilirubin, thereby increasing its water-solubility.

a strong positive correlation was shown between light irradiance and decrease in TSB during the first 24h of treatment, and no "saturation point" was found.[205] Phototherapy is most efficient in the first 24-48 hours of treatment. The declining efficacy after 48 hours is probably related to the fact, that configurational photoisomers that have been reverted to UCB undergo EHC, and increase the UCB load to be cleared by the liver. In addition, photoisomers have a strong tendency to relapse back in their original structure, which strongly limits the efficacy of phototherapy. If necessary, double-sided phototherapy can be used in order to increase the therapeutic effect. Double-sided phototherapy involves a conventional overhead lamp plus a light-emitting blanket, a so-called "bili-blanket".[206]

Phototherapy was introduced almost 60 years ago, and since then no serious long-term side effects, such as skin cancers, have been observed. Short-term phototherapy is generally considered safe[190], but side effects have been reported. Phenomena that have been attributed to, or associated with, phototherapy include retinal damage if eyes are not shielded from light by eye patches[207], diarrhea

and decreased gut transit time[208,209], temperature instability, and patent ductus arteriosus.[210,211] Phototherapy during cholestasis may produce the "bronze baby syndrome", due to accumulation of photoproducts that would normally have been excreted *via* the bile.[212] Consequently, the skin, serum, and urine develop a grayish-brown discoloration. This discoloration disappears if the cholestasis resolves or if the phototherapy is discontinued.[213,214] In neonates with cholestatic jaundice rare purpuric and bullous eruptions have been observed during phototherapy.[215,216] Phototherapy may also induce insensible water loss.[217] Finally, the erythropoietic porphyrias and photosensitizing drugs are absolute contraindications to phototherapy.[190,218]

Long-term phototherapy, as needed by patients with Crigler-Najjar disease type I, has considerable disadvantages. Phototherapy becomes less effective with age, due to a decrease in surface area to body mass ratio[130,131], due to a large tissue reservoir of UCB[131], due to skin alterations[125,129], and due to a diminishing compliance to the intensive phototherapy regimen, which has a profound impact on the quality of (social) life.[129] On the other hand, long-term treatment, *i.e.* years of phototherapy, in individual patients did not show any serious side-effects.

Exchange transfusion

Exchange transfusion is momentarily the only effective alternative to phototherapy in patients with severe unconjugated hyperbilirubinemia. With this technique, approximately 85% of the circulating red blood cells, depending on the volume actually exchanged, will be replaced by a mixture of red cells and plasma from a donor. Plasma UCB concentrations will generally be reduced by 50%, although this amount varies according to the severity of the ongoing hemolysis, and the amount of bilirubin that re-enters the circulation from the tissues.[72] This re-entry occurs due to the diffusion of UCB from the tissue pool into the plasma pool and decreases the risk of bilirubin-induced neurotoxicity. Exchange transfusion is especially effective during immune-mediated hemolytic disease, since it also decreases the amount of antibodies that target the erythrocytes from the circulation.[72]

Indications for exchange transfusion include dangerously high or rapidly rising plasma UCB concentrations despite phototherapy, progressive anemia due to hemolysis, or symptoms and signs of acute bilirubin-encephalopathy.[166,219] Exchange transfusions carry considerably higher risks than phototherapy. The mortality rate from the procedure is 0.3-2.0%. Significant morbidity occurs in 5-12% of exchange transfusions.[220-222] Complications include cardiac arrest, thrombosis of the portal vein, graft versus host disease, coagulopathies, hypoglycemia, hypocalcaemia, necrotizing enterocolitis, and transmission of infectious diseases.[220-224] Fortunately, the need for exchange transfusions has been greatly reduced since the introduction of phototherapy.[196,225]

Albumin administration

An increasing amount of evidence obtained from animal experiments and from clinical studies suggests, that B_f predicts bilirubin induced brain damage more accurately than TSB concentrations.

Whereas TSB reflects the total serum bilirubin load, concentrations of B_f might thus better reflect the risk of brain tissue exposure to bilirubin. Therefore, albumin administration prior to exchange transfusion, could be a useful strategy in decreasing B_f, due to an increase of the binding of UCB to albumin.[226] The efficacy of albumin administration in combination with an exchange transfusion has not yet been established. In chapter 3, we optimized a hyperbilirubinemic animal model for exchange transfusion and we show the results of combining exchange transfusion with phototherapy and/or albumin administration.

7.2. Treatment options for Crigler-Najjar patients

Liver transplantation

Currently, liver transplantation is the only definitive treatment for Crigler-Najjar disease type I. Several patients with Crigler-Najjar disease type I have undergone liver transplantations.[129,227-229] Successful liver transplantation effectively restores UGT1A1 activity, which results in low or normal plasma UCB concentrations, and eliminates the need for phototherapy.[129] However, liver transplantation remains a major, high-risk procedure, with a one year survival between 85% and 90%.[230,231] Therefore, the benefits of transplantation need to be weighted against the complications and risks. Possible complications include rejection, infection, bleeding, thrombosis and biliary complications.[230,231] To reduce the risk of rejection, patients receive life-long immunosuppressive therapy, which increases the risk of lymph-proliferative diseases and infections, and has side effects as nephrotoxicity and hyperlipidemia.

Two types of transplantation have been used. In orthotopic liver transplantation (OLT) the patient's own liver is replaced by a donor liver. In auxiliary liver transplantation (part of) the patient's own liver is left *in situ* and is supported by a donor graft.[232] The theoretical advantage of the latter procedure is that, if gene therapy becomes available in the future, this could still be applied to the native liver, allowing possible withdrawal of immunosuppression. Presently, the OLT procedure is the common procedure if a transplant is performed.

Hepatocyte transplantation

Hepatocyte transplantation is theoretically an attractive alternative to liver transplantation, since the architecture of the liver is normal in patients with Crigler-Najjar disease type I. Hepatocyte transplantation might be less invasive and thus safer than liver transplantation. Also, a complete liver transplant is unnecessary to correct Crigler-Najjar disease type I, since only partial replacement of UGT1A1 activity is required for correction of the phenotype.[233] Several techniques of hepatocellular transplantation have been investigated in the Gunn rat.[234] Generally, the best results were obtained when hepatic injury was caused prior to infusion of congenic hepatocytes *via* the liver, spleen, portal vein, or *via* intraperitoneal injection. Hepatic damage provided a regenerative stimulus for the engrafting cells, which resulted in a significant lowering in plasma UCB for up to 12 months.[234]

In patients, the first hepatocyte transplant was performed in a 10-year old patient with Crigler-Najjar disease type I, in which the infusion of 7.5 x 10^9 liver cells *via* the portal vein decreased plasma bilirubin levels for up to 11 months.[235] However, the restoration in enzyme activity (~5% of normal) was not sufficient to eliminate the eventual need for liver transplantation. Since this initial report, six additional patients have been treated with hepatocyte or hepatocyte progenitor cell transplantation, often with multiple infusions.[234,236-239] In all these studies the long-term follow up (if reported) showed a rebound of plasma bilirubin concentrations to pre-treatment levels after five to six months, which was probably due to unfavorable immune reactions. Thus, the ultimate need for OLT has not been changed in these patients. Currently, hepatocyte transplantation is thus still hampered by its transient therapeutic effect. Also, immunosuppressive therapy is still required and donor livers, or fetal hepatic progenitor cells, are still required to isolate cells.[234,236-239] Nevertheless, the technique appears to be relatively safe, and could be used to bridge the gap to OLT in Crigler-Najjar patients.

Gene therapy

As stated above, Crigler-Najjar is caused by a deletion in a single gene, and only a partial replacement of the UGT1A1-enzyme activity would be enough to significantly lower plasma UCB concentrations and successfully treat the disease. The option of partial replacement of the enzyme makes the disease theoretically an ideal candidate for gene therapy. Numerous strategies of gene transfer, using viral and non-viral vectors, have been developed in the Gunn rat model, and resulted in a long-term correction in plasma UCB levels. However, vector toxicity and concerns about long-term safety have so far prevented the use of gene therapy in patients.

The first gene transfer strategy evaluated in Gunn rats made use of retroviral vectors.[240,241] Although these vectors efficiently integrated UGT1A1 into the host's genome, the technique was not very effective in larger animals. This could be due to the inability of retroviruses to infect non-dividing cells.[242] Another approach involved the use of adenoviral vectors that target the liver and do have the ability to infect non-dividing cells. First-generation adenoviral vectors could only correct UGT1A1 for a limited time, probably related to the fact that they were not incorporated into the host's genome.[243,244] Several strategies to prolong the duration of transgene expression have been explored, including induction of tolerance[245] and expression of immunomodulatory molecules.[246] However, acute toxicity and immunogenicity of viral proteins were a major disadvantage of these vectors. Subsequently, vectors with a lower immunogenicity were developed, in which virtually all viral genes have been deleted.[242] These second-generation adenoviral vectors corrected plasma UCB levels for over two years in Gunn rats.[247] Immunogenicity has also been decreased by inducing tolerance for adenoviruses, and by using adenoviral vectors that express immune-modulating molecules.[245,246,248] Finally, lenti-viruses and SV40 viruses have been used as vectors, which allowed incorporation of UGT1A1 into the genome of non-dividing cells. Although results in Gunn rats seem encouraging, these vectors do not specifically localize to the liver and must be administrated

via the portal vein.[249-251] Non-viral strategies based on chimeraplasty[252], liposomes[253], or plasmids[248,254] have also been evaluated. Non-viral vectors use receptor-mediated endocytosis to transfer UGT1A1 into the hepatocyte.[255,256] Finally, ex-vivo gene therapy with transplantation of manipulated fibroblasts corrected the gene defect but resulted in animals developing tumors.[257] The results of gene therapy thus seem promising in Gunn rats. Currently, however, the results of clinical trials must be awaited before any of these strategies can be applied in Crigler-Najjar patients.

8. SCOPE OF THIS THESIS

This thesis focuses on new diagnostic and therapeutic strategies for severe unconjugated hyperbilirubinemia. In the first postnatal week, a transient increase in plasma unconjugated bilirubin (UCB) concentrations occurs in 70% of term, and in almost all preterm infants. This so called physiological jaundice is due to an immature hepatic conjugation, an increased UCB production because of the increased erythrocyte turnover, and an increased enterohepatic circulation (EHC) of UCB. This physiological jaundice is considered a benign phenomenon, which even might be beneficial due to the anti-oxidant properties of UCB.[8] However, pathological jaundice, with potentially toxic increase in plasma UCB concentrations, is mostly due to an exaggeration of the mechanisms that cause physiological jaundice. Exaggeration occurs in conditions such as neonatal hemolytic jaundice (due to excessive UCB production from heme), and Crigler-Najjar disease.

As stated above, Crigler-Najjar disease is characterized by a genetically absent (type I) or decreased (type II) activity of the hepatic enzyme bilirubin-uridine-diphosphoglucuronosyltransferase (UGT1A1).[126,127] This enzyme catalyzes bilirubin conjugation in the hepatocyte, and greatly enhances its biliary secretion and subsequent fecal excretion.[39]

Severe unconjugated hyperbilirubinemia can cause bilirubin-induced neurological damage and kernicterus, resulting in physical and mental handicaps or even death.[86] This damage is mediated by the ability of "free" bilirubin (B_f), the small (<1%) fraction of UCB not bound to plasma proteins, to cross the blood-brain barrier.[26-28,88,258]

Hyperbilirubinemia is classically quantified, and thus monitored, *via* total serum bilirubin (TSB) concentrations. Although, TSB determination is the current gold standard, one disadvantage is the need of repeated invasive blood sampling. A possible alternative is the non-invasive and painless screening method for hyperbilirubinemia, transcutaneous bilirubin (TcB) measurement. This methodology has been shown to correlate reasonably well with TSB up to a certain level of bilirubin concentrations, and can be used for screening jaundice in human newborns.[259,260]

Presently, the standard treatment for hyperbilirubinemia is phototherapy (PT) and/or exchange transfusion (ET). Phototherapy is generally effective, but in some neonates the plasma bilirubin concentrations become dangerously high or rise rapidly despite PT. In these patients, PT might

fail to prevent bilirubin-induced brain damage. Furthermore, Crigler-Najjar patients may need up to 16h of treatment per day. In spite of this intensive regimen, up to 25% of the Crigler-Najjar patients will eventually develop brain damage.[129,130] Exchange transfusions have been considered as a "rescue treatment", based on its invasiveness and related risks. Significant morbidity, and even mortality, has been reported.[220-222]

These considerations underline the ambition to develop better diagnostic and therapeutic options.

Therapy to prevent neurotoxicity should decrease brain rather than plasma bilirubin concentrations. Free bilirubin, the unbound fraction of UCB, is able to diffuse from the plasma into the tissue pool.[12,28] Decreasing B_f in plasma could prevent bilirubin deposition in the brain. Theoretically, albumin administration could achieve this goal, by providing more binding sides for B_f. In **chapter 2** of this thesis we determined the effect of PT and PT + albumin administration on UCB, B_f and brain bilirubin concentrations during permanent and acute hemolytic jaundice in Gunn rats. The Gunn rat is the well-established animal model for Crigler-Najjar disease type I.

Phototherapy and ET are cornerstones in treatment of acute severe unconjugated hyperbilirubinemia. Studies to improve ET efficacy and/or to minimize its risks have been hampered by the relatively low application rate of ET in humans and by the lack of an *in vivo* model system. The absence of an appropriate animal model has also prevented to determine the efficacy of adjunct or alternative treatment options such as albumin administration. In **chapter 3** we optimized an *in vivo* model for ET and determined the most effective treatment (combination) of ET, PT and albumin administration.

To study neonatal jaundice, we considered it important to perform treatment experiments in Gunn rat pups, which resemble the clinical situation in neonates better than adult Gunn rats. Free bilirubin can induce neurotoxicity, including impairment of the auditory system, which can be assessed by brainstem auditory evoked potentials (BAEPs). **Chapter 4** describes the effects of albumin administration on BAEPs as a qualitative measure, and brain bilirubin levels as a quantitative measure in Gunn rat pups. We determined plasma UCB concentrations, plasma B_f concentrations, total brain bilirubin content, and free brain bilirubin content in two Gunn rat pup models of acute hyperbilirubinemia. The first model is based on hemolysis due to a phenylhydrazine injection. The second model is based on bilirubin displacement from albumin by a sulfadimethoxine injection.

Developing hyperbilirubinemia in preterm infants is diagnosed and monitored *via* TSB concentrations. As discusses above, TcB measurements are a possible non-invasive and painless screening method, and thus an alternative for invasive blood sampling. Peak bilirubin levels in neonates can be found between postnatal days 2-7, and in rats between postnatal days 15-18. Prevention of this bilirubin peak might be achieved by increasing the fecal excretion of bilirubin.

In previous studies in adult Gunn rats, we demonstrated that polyethylene glycol (PEG) and ursodeoxycholic acid (UDCA) can prevent hyperbilirubinemia by accelerating the fecal excretion of bilirubin. However, the preventive capacity of these strategies for neonatal jaundice is unknown. In the experiment described in **chapter 5** we evaluated the preventive potency of PEG and UDCA during the hyperbilirubinemic peak in Gunn rat pups.

It is known that B_f is more closely related to bilirubin neurotoxicity than TSB. In the clinical setting, however, B_f cannot (yet) be measured routinely. The bilirubin/albumin (B/A) ratio has been put forward as an alternative for estimating B_f. Accordingly, high B/A ratios are considered risk factors for bilirubin neurotoxicity.
In **chapter 6**, we made the step from bench to bed-side and described the postnatal courses of B_f and the B/A ratio in preterm infants, and evaluated the relationship between these parameters.

Finally, **chapter 7** contains a general discussion of the different studies. The new insights are related to previously available literature, and placed in a clinical and experimental framework. The implications of the present findings and the future perspectives, both with respect to clinical and scientific developments, are discussed.

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Chapter 2

BEYOND PLASMA BILIRUBIN: THE EFFECTS OF PHOTOTHERAPY AND ALBUMIN ON BRAIN BILIRUBIN LEVELS IN GUNN RATS

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ABSTRACT

Background and Aims: Severe unconjugated hyperbilirubinemia, as occurs in Crigler-Najjar disease and neonatal jaundice, carries the risk of neurotoxicity. This neurotoxicity is related to the ability of free bilirubin (B_f), the fraction of bilirubin that is not bound to plasma proteins, to translocate into the brain. We hypothesized that albumin treatment would lower the B_f fraction, and thus decreases bilirubin accumulation in the brain.

Methods: We treated chronic (*e.g.* as a model for Crigler-Najjar disease) and acute hemolytic (*e.g.* as a model for neonatal jaundice) moderate hyperbilirubinemic Gunn rats with phototherapy, human serum albumin (HSA) or phototherapy+HSA.

Results: In the chronic model, adjunct HSA increased the efficacy of phototherapy; it decreased plasma B_f and brain bilirubin by 88% and 67%, respectively ($p<0.001$). In the acute model, adjunct HSA also increased the efficacy of phototherapy; it decreased plasma B_f by 76% ($p<0.001$) and completely prevented the hemolysis-induced deposition of bilirubin in the brain. Phototherapy alone failed to prevent the deposition of bilirubin in the brain during acute hemolytic jaundice.

Conclusion: We showed that adjunct HSA treatment decreases brain bilirubin levels in phototherapy-treated Gunn rats. We hypothesize that HSA decreases these levels by lowering B_f within the plasma. Our results support the feasibility of adjunct albumin treatment in patients with Crigler-Najjar disease or neonatal jaundice.

INTRODUCTION

Crigler-Najjar patients and hemolytic neonates suffer from unconjugated hyperbilirubinemia.[1] Severe unconjugated hyperbilirubinemia can lead to brain damage. This damage is mediated by the ability of "free" bilirubin (B_f), the small (<1%) fraction of unconjugated bilirubin (UCB) not bound to plasma proteins, to cross the blood-brain barrier (BBB).[2-6] Within the brain, UCB disrupts several vital cell functions and induces apoptosis and necrosis. Bilirubin-induced neurotoxicity may eventually lead to kernicterus and even death.[3,7,8]

Severe unconjugated hyperbilirubinemia is conventionally treated by phototherapy, which converts UCB into photo-isomers that can readily be excreted in the bile.[9] Phototherapy, however, has some disadvantages. Crigler-Najjar patients, who suffer from permanent inherited unconjugated hyperbilirubinemia, may need up to 16h of treatment per day. In spite of this intensive regimen, up to 25% of these patients will eventually develop brain damage.[10,11] Phototherapy is more effective during neonatal hemolytic jaundice, but may still require additional, potentially dangerous, exchange transfusions.[12] The efficacy of phototherapy is often estimated by its hypobilirubinemic effect. Plasma bilirubin levels, however, correlate poorly with the occurrence of brain damage in individual patients.[6] The reason for this poor correlation lies in the inability of protein-bound bilirubin (>99% of total plasma bilirubin) to leave the circulation.[2,3,5,6] Only B_f is able to translocate across the BBB, and thus plays a key role in the pathogenesis of bilirubin-induced brain damage. Free bilirubin concentrations, however, are not routinely evaluated in phototherapy-treated patients. The main reason for this lies in the inaccuracy of the commercial B_f test, most notably caused by a 42-fold sample dilution that alters bilirubin-albumin binding.[13]

We reasoned that decreasing B_f in the plasma could prevent bilirubin deposition in the brain. Human serum albumin (HSA) infusion could, theoretically, achieve this goal by providing additional binding sites for B_f in the plasma. Interestingly, HSA treatment has been used in severely jaundiced neonates.[14-18] Its efficacy, however, has been difficult to establish. This difficulty is due to the obvious inability to measure bilirubin brain levels in humans, but also to the aforementioned inaccuracy of the commercially available B_f test. Recently, Zelenka *et al.* developed a highly sensitive method for tissue bilirubin determinations, while Ahlfors *et al.* developed an automated B_f test with minimal sample dilution.[13,18] We now use these techniques to evaluate the effect of HSA treatment on plasma B_f and brain bilirubin levels in two well-established animal models. As a moderate chronic model, resembling patients with Crigler-Najjar disease, we treated adult Gunn rats with long-term phototherapy, HSA, or with phototherapy+HSA.[19] As an acute model, resembling severe hemolytic jaundice, we induced hemolysis by 1-acetyl-2-phenyl-hydrazine (APHZ) in adult Gunn rats, and then treated these animals for 48h with phototherapy, HSA, or phototherapy+HSA.[20] We demonstrate that HSA treatment decreases plasma B_f and brain bilirubin levels in phototherapy-treated Gunn rats, both during chronic and acute jaundice. We speculate that HSA and phototherapy work *in tandem*: HSA binds B_f within the plasma, and phototherapy

then promotes its excretion *via* the bile. Our results underline the need to evaluate the use of HSA as adjunct to phototherapy in randomized clinical trials.

ANIMALS, MATERIALS, AND METHODS

Animals

Homozygous male Gunn rats, the animal model for Crigler-Najjar disease type I (RHA/jj; 225-340g, aged 10-12 weeks), were obtained from our breeding colony, kept in an environmentally controlled facility, and were fed *ad libitum* with free access to water. Food intake, fluid intake, and body weight were determined regularly. The Animal Ethics Committee of the University of Groningen (Groningen, The Netherlands) approved all experimental protocols.

Materials

Hope Farms B.V. (Woerden, The Netherlands) produced the semi-synthetic control diet (code 4063.02), containing 13 energy% fat and 5.2 wt% long-chain fatty acids. In previous studies, we have noticed that diet and diet-composition influence plasma bilirubin levels. We used the same semi-synthetic control diet as in previous studies to enhance the reproducibility, and to allow comparison between studies.[21,22] Gunn rats were fed this diet during a 5-week run-in period, to ensure steady-state conditions, and during the experiments. HSA (Albuman®; 200 g/L, fatty acid free) was purchased from Sanquin (Amsterdam, The Netherlands). APHZ, horseradish peroxidase type 1, D-glucose, glucose oxidase, hydrogen peroxide and UCB were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Commercial UCB was further purified according to the method of Ostrow *et al.*[23] Phototherapy was administered continuously to Gunn rats (shaven on flank and back) *via* two blue phototherapy lamps (Philips, TL-20W/03T) suspended in a reflective canopy 20 cm above the cage. The phototherapy dose (17 μW/cm^2/nm; 380-480 nm) was measured by an Elvos-LM-1010 Lux meter at 20 cm distance.[24]

Methods

Permanent unconjugated hyperbilirubinemia

Adult Gunn rats were randomized to receive either no treatment (n=13) or phototherapy (17 μW/cm^2/nm; n=14) for 16 days. At time (t)=14 days, we randomized the animals to receive either no treatment (n=7), phototherapy+NaCl 0.9% (17 μW/cm^2/nm; n=7), HSA (2.5 g/kg; n=6), or phototherapy+HSA (n=7), for another 48h. NaCl 0.9% (control/sham) and HSA were administered as a single *i.p.* injection at t=14 days. We determined plasma bilirubin concentrations from tail vein blood at t=0, 14, and 16 days, and determined plasma B_f at t=16 days under isoflurane anesthesia. After 16 days, all animals were exsanguinated *via* the descending aorta and flushed *via* the same port with 100-150 ml NaCl 0.9% under isoflurane anesthesia. The brain, liver, and aliquots of visceral fat were subsequently harvested for the determination of tissue bilirubin levels. These

samples were rinsed two times in phosphate buffered saline, snap frozen in liquid nitrogen, and immediately stored (wrapped in aluminum foil) at -80 °C until analysis.[25]

Acute unconjugated hyperbilirubinemia

Adult Gunn rats received a single APHZ injection *i.p.* (15 mg/kg BW; t=-24h) to induce hemolysis. We then randomized these animals after 24h (t=0h) to receive either no treatment (n=6), phototherapy+NaCl 0.9% (17 μW/cm^2/nm; n=6), HSA (2.5 g/kg; n=6), or phototherapy+HSA (n=6) for another 48h. NaCl 0.9% (control/sham) and HSA were administered as a single *i.p.* injection at t=0h. We determined plasma bilirubin, B_f and albumin concentrations from tail vein blood at t=-24, -12, 0, 12, 24, 36, and 48h under isoflurane anesthesia. Hemoglobin (Hb), reticulocyte count and hematocrit (Ht), were determined at t=-24h and t=48h. After 48h, all animals were exsanguinated and brain, liver, and visceral fat samples were subsequently harvested for the determination of tissue bilirubin levels, as described above.[25]

Plasma analysis

Blood samples were protected from light, stored at -20 °C under argon directly after collection and processed within two weeks. UCB concentrations were determined by routine spectrophotometry on a P800 unit of a modular analytics serum work area from Roche Diagnostics Ltd. (Basel, Switzerland). Hb, Ht, and reticulocytes were determined on a Sysmex XE-2100 hematology analyzer (Goffin Meyvis, Etten-Leur, The Netherlands). We previously found in Gunn rats that the total bilirubin concentration, measured by spectrophotometry, equals the total UCB concentration, measured by high-liquid performance chromatography (HPLC) after chloroform extraction (coefficient of variation: ~5%).[21,22] B_f was determined using a Zone Fluidics system (Global Flopro, Global Fia Inc, WA), as previously described by Ahlfors *et al.*[13]

Tissue bilirubin analysis

Tissue bilirubin content was determined using HPLC with diode array detector (Agilent, Santa Clara, CA, USA) as described earlier.[25] Briefly, 300 pmol of mesobilirubin in DMSO (used as an internal standard) was added, and samples were homogenized on ice. Bile pigments were then extracted into chloroform/hexane (5:1) solution at pH 6.0, and subsequently extracted in a minimum volume of methanol/carbonate buffer (pH 10.0) to remove contaminants. The resulting polar droplet (extract) was loaded onto a C-8 reverse phase column (Phenomenex, Torrance, CA, USA) and separated pigments were detected at 440 nm. The concentration of bilirubin was calculated as nmol/g of wet tissue weight. All steps were performed under dim light in aluminum-wrapped tubes. We did not specifically measure bilirubin deposition in the brain nuclei, but relied on total tissue bilirubin measurements.

Statistical analysis

Normally distributed data that displayed homogeneity of variance (by calculation of Levene's statistic), were expressed as mean ± SD, and analyzed with parametric statistical tests. Analysis of variance (ANOVA) with post-hoc Tukey correction was performed for comparisons between groups, and the Student *t* test for comparison of paired data within groups. The level of significance was set at $p<0.05$. Analyses were performed using PASW Statistics 17.0 for Mac (SPSS Inc., Chicago, IL).

RESULTS

Chronic unconjugated hyperbilirubinemia

Adjunct HSA treatment decreases plasma B_f concentrations

We first treated permanently jaundiced Gunn rats, as a model for Crigler-Najjar disease, with routine phototherapy, HSA, or phototherapy+HSA for 16 days. Figure 1A shows that phototherapy and phototherapy+HSA decreased plasma UCB concentrations to a similar extent (46% and 54% at t=16 days, respectively), compared with untreated controls ($p<0.001$). HSA alone increased plasma UCB concentrations with 65% compared with controls ($p<0.001$). Figure 1B shows that phototherapy, HSA and phototherapy+HSA decreased plasma B_f concentrations by 55%, 54% and 88%, respectively ($p<0.001$). HSA alone decreased the unbound fraction of UCB from 0.08% to 0.02% (-71%; $p<0.001$), compared with controls. Adjunct HSA lowered plasma B_f levels by 33%, compared with phototherapy alone ($p<0.01$). Mean growth rates did not differ significantly between experimental and control groups during the experiment (data not shown).

Adjunct HSA treatment decreases brain bilirubin levels

Figure 1C shows that phototherapy, HSA alone, and phototherapy+HSA decreased brain bilirubin levels by 45%, 35% and by 67%, respectively ($p<0.01$), compared with untreated controls. Adjunct HSA thus lowered brain bilirubin levels by an additional 22% (NS), compared with phototherapy alone. Adjunct HSA significantly decreased hepatic bilirubin levels by an additional 33% ($p<0.01$), compared with phototherapy alone (Figure 5A), but failed to induce a significant additive decrease in visceral fat bilirubin levels (Figure 5B).

The correlation between B_f and brain bilirubin levels

Figure 2A illustrates the poor correlation between plasma UCB concentrations and brain bilirubin levels ($y=0.0037x+1.52$; $R^2=0.17$; $p<0.05$). The HSA group, with bilirubin levels above 300 µmol/L, seemed mainly responsible for this poor correlation. Figure 2B shows that plasma B_f concentrations correlated well with brain bilirubin levels ($y=0.013x+1.00$; $R^2=0.74$; $p<0.001$).

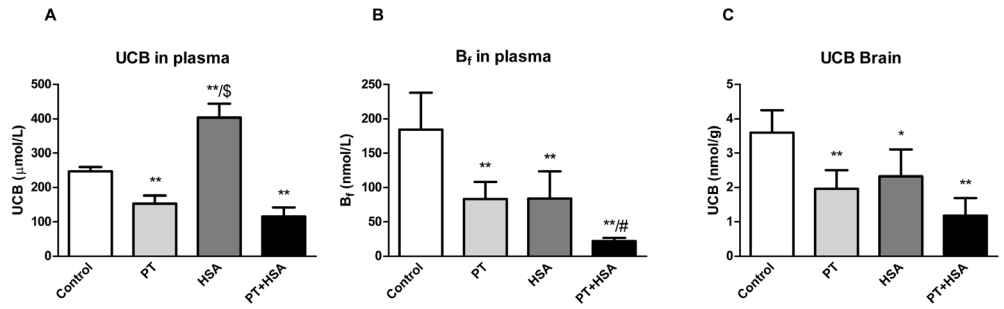

Figure 1. Treatmenteffects during chronic hyperbilirubinemia
Effects of no treatment (controls), routine phototherapy (PT), human serum albumin (HSA), or phototherapy+HSA on plasma UCB (**A**), plasma B_f (**B**), and brain bilirubin levels (**C**) in Gunn rats at t=16 days. Adult Gunn rats were randomized to receive either no treatment or phototherapy (17 µW/cm²/nm) for 16 days. After t=14 days, we randomized the animals to receive no treatment, phototherapy (17 µW/cm²/nm), HSA (2.5 g/kg), or phototherapy+HSA for another 48h. *p<0.01, **p<0.001 compared with controls. #p<0.05, $p<0.001 compared with phototherapy alone.

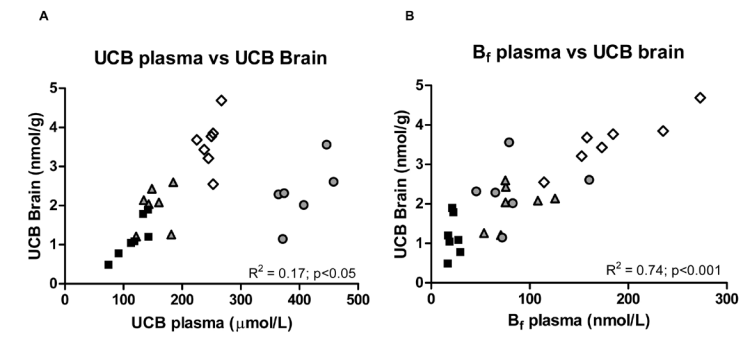

Figure 2. Correlations between plasma UCB, B_f, and brain bilirubin levels during chronic hyperbilirubinemia.
The correlation between plasma UCB and brain bilirubin levels (**A**), and the correlation between plasma B_f and brain bilirubin levels (**B**) in Gunn rats at t=16 days. Adult Gunn rats were randomized to receive either no treatment or phototherapy (17 µW/cm²/nm) for 16 days. After t=14 days, we randomized the animals to receive no treatment, phototherapy (17 µW/cm²/nm), human serum albumin (HSA: 2.5 g/kg), or phototherapy+HSA for another 48h. ◇ control, △ phototherapy, ○ HSA, ■ phototherapy+HSA.

Acute unconjugated hyperbilirubinemia

APHZ induces comparable hemolysis in all treatment groups

As a model for acute unconjugated hyperbilirubinemia we then used APHZ to induce hemolytic jaundice in Gunn rats. APHZ administration induced a comparable hemolysis in all groups, as indicated by the similar changes in Hb, Ht, and reticulocyte levels (Figure 3A-C). Figure 3D shows that a single *i.p.* HSA injection increased plasma albumin within 12h (+34% and +40% in HSA and phototherapy+HSA-treated animals, respectively), compared with untreated controls. Mean growth rates did not differ significantly between experimental and control groups during the experiment (data not shown).

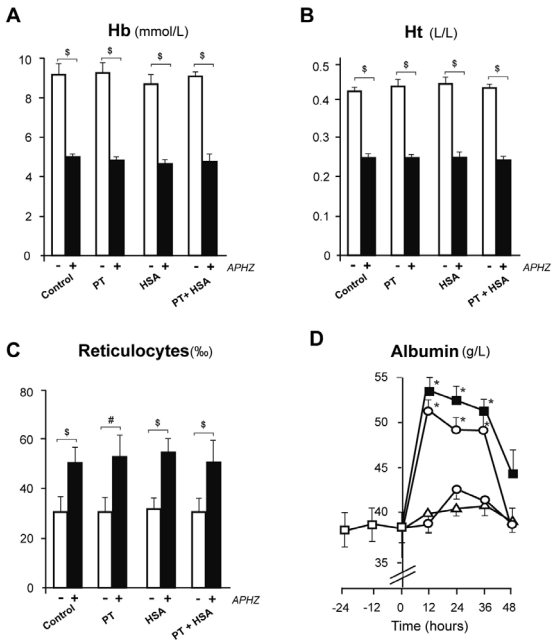

Figure 3. Effect of APHZ injection on Hb, Ht, reticulocytes, and the effect of HSA injection on plasma albumin levels.

Effects of APHZ injection on Hb (**A**), Ht (**B**), and reticulocyte (**C**) levels, and the effect of human serum albumin (HSA) injection on plasma albumin levels (**D**) in Gunn rats. Adult Gunn rats received APHZ *i.p.* to induce hemolysis, and were randomized after 24h to receive no treatment, phototherapy (17 μW/cm^2/nm), human serum albumin (HSA: 2.5 g/kg), or phototherapy+HSA for 48h. *p<0.001, compared with controls. #p<0.01; $p<0.001, compared with pre-treatment values at t=0h.

◇ control, △ phototherapy, ○ HSA, ■ phototherapy+HSA.

Adjunct HSA treatment decreases plasma UCB concentrations

We treated the hemolytic Gunn rats with routine phototherapy, HSA, or phototherapy+HSA for 48h. Figure 4A shows that phototherapy and phototherapy+HSA both decreased the severity of hemolytic unconjugated hyperbilirubinemia, compared with untreated hemolytic controls. Phototherapy decreased plasma UCB concentrations by 14% at t=36h (p<0.01), while phototherapy+HSA decreased these concentrations by at least 29% from t=36h onwards (p<0.001). Adjunct HSA thereby lowered plasma bilirubin levels by an additional 14-16%, compared with phototherapy alone (p<0.05). HSA alone failed to decrease plasma UCB concentrations.

Adjunct HSA treatment decreases plasma B_f concentrations

Figure 4B shows that phototherapy decreased plasma B_f concentrations by 31% at t=48h (p<0.05), compared with controls, while phototherapy+HSA decreased these concentrations by at least 41% from t=12h onwards (p<0.001). Adjunct HSA thereby lowered plasma B_f concentrations by an additional 25-47%, respectively, compared with phototherapy alone (p<0.05). HSA alone failed to decrease plasma B_f concentrations, in spite of a transient drop in B_f concentrations during the first 24h of treatment.

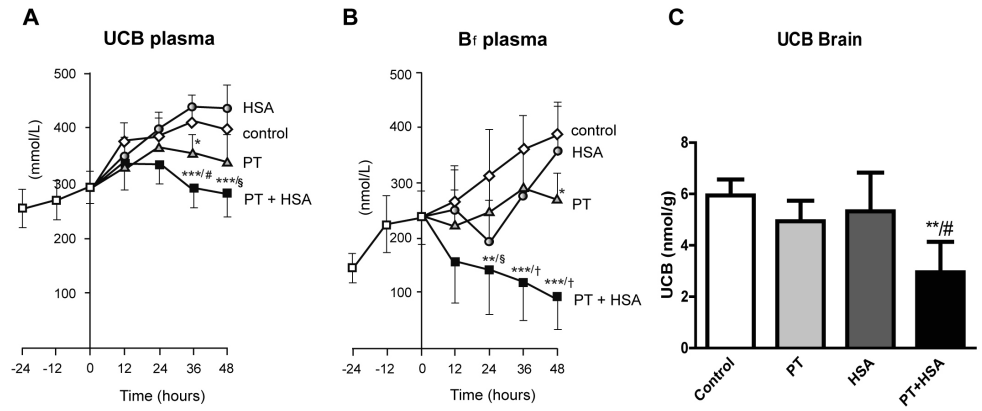

Figure 4. Treatementeffects during acute hyperbilirubinemia.

Effects of no treatment (controls), routine phototherapy (PT), human serum albumin (HSA), or phototherapy+HSA on plasma UCB (**A**), plasma B_f (**B**), and brain bilirubin levels (**C**) at t=48h in hemolytic Gunn rats. Adult Gunn rats received APHZ *i.p.* to induce hemolysis, and were randomized after 24h to receive no treatment, phototherapy (17 μW/cm^2/nm), HSA (2.5 g/kg), or phototherapy+HSA for 48h. Plasma bilirubin concentrations were similar in all groups during the 24h-run in period after APHZ injection. *p<0.05; **p<0.01; ***p<0.001, compared with controls. #p<0.05; §p<0.01; †p<0.001, compared with phototherapy alone.

Adjunct HSA treatment decreases brain bilirubin levels

Figure 4C shows that phototherapy alone, and HSA alone both failed to decrease brain bilirubin levels. Combining phototherapy with HSA, however, resulted in a 50%-decrease in brain bilirubin levels, compared with untreated hemolytic controls ($p<0.001$). Adjunct HSA thereby decreased brain bilirubin levels to 2.9±1.2 nmol/g, which was comparable with the brain bilirubin content of non-hemolytic control animals (3.6±0.7 nmol/g; Figure 1C). Adjunct HSA thus completely prevented the deposition of bilirubin in the brain during hemolytic jaundice.

Adjunct HSA also significantly decreased hepatic bilirubin levels by an additional 36% ($p<0.01$), compared with routine phototherapy (Figure 5C), and phototherapy+HSA was the only treatment that significantly decreased bilirubin levels in visceral fat, compared with controls (-41%, $p<0.05$; Figure 5D).

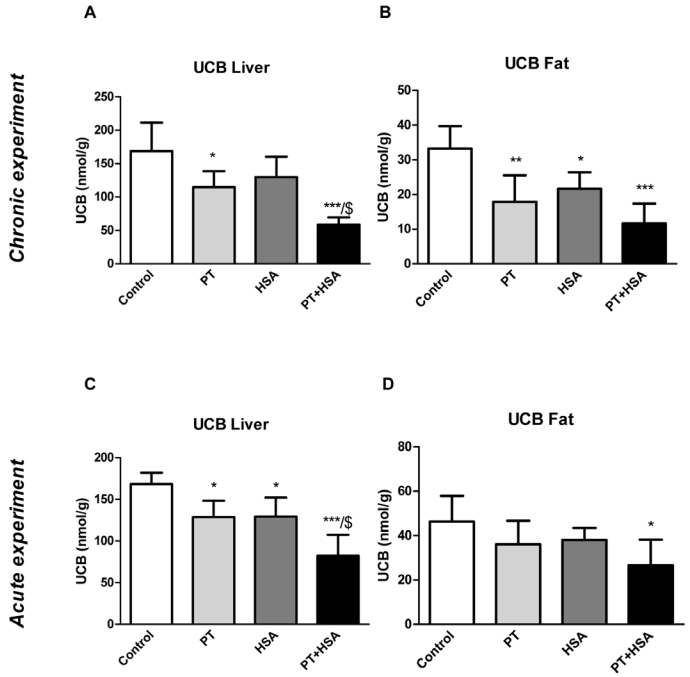

Figure 5. Tissue bilirubin levels.
A/B: effects of no treatment (controls), routine phototherapy (PT), human serum albumin (HSA), or phototherapy+HSA on liver (**A**) and visceral fat (**B**) bilirubin levels in non-hemolytic (chronic experiment) Gunn rats. **C/D**: effects of no treatment (controls), routine phototherapy (PT), HSA, or phototherapy+HSA on liver (**C**) and visceral fat (**D**) bilirubin levels in hemolytic (acute experiment) Gunn rats. For experimental setup, we kindly refer to the Methods section. *p<0.05; **p<0.01; ***p<0.001, compared with controls. $p<0.01, compared with phototherapy alone.

The correlation between plasma B_f and brain bilirubin levels

Figure 6A shows the correlation between plasma bilirubin and brain bilirubin levels during acute jaundice. Figure 6B shows that plasma B_f correlates reasonably well with brain bilirubin levels in hemolytic Gunn rats ($y=0.0078x+2.63$; $R^2=0.48$; $p<0.001$).

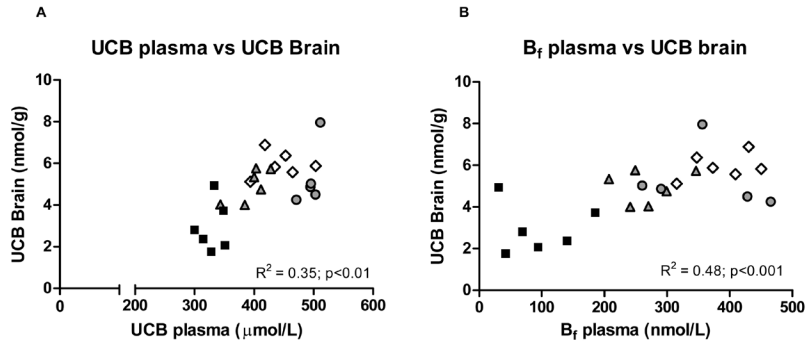

Figure 6. Correlations between plasma UCB, B_f, and brain bilirubin levels during acute hyperbilirubinemia.
The correlation between plasma UCB and brain bilirubin levels (**A**), and the correlation between plasma B_f and brain bilirubin levels (**B**) in Gunn rats at t=48h. Adult Gunn rats received APHZ *i.p.* to induce hemolysis, and were randomized after 24h to receive no treatment, phototherapy (17 μW/cm²/nm), human serum albumin (HSA: 2.5 g/kg), or phototherapy+HSA for 48h. ◇ control, ▲ phototherapy, ○ HSA, ■ phototherapy+HSA.

DISCUSSION

In this study we demonstrate that HSA effectively decreases brain bilirubin levels in phototherapy-treated Gunn rats. The decrease was apparent during both chronic and acute hemolytic jaundice. Our results support the feasibility of HSA treatment as adjunct to phototherapy in Crigler-Najjar disease and neonatal jaundice.

The rationale behind HSA treatment is based on the premises that B_f translocates into the brain and, secondly, that *i.v.* albumin prevents this translocation by binding to B_f within the plasma. The role of B_f translocation became apparent in the 1950s, when sulfisoxazole-treated neonates developed kernicterus in the presence of unusually low plasma bilirubin concentrations.[26,27] It was soon discovered that sulfisoxazole displaced UCB from albumin, which first suggested the importance of the non-albumin bound UCB fraction.[28] Since then, many studies have supported the critical role of B_f in the pathogenesis of bilirubin-induced brain damage.[2,3,5,6] These studies also demonstrated that plasma B_f levels increased proportionally as the plasma albumin binding affinity or capacity decreased, or during inflammation. Ahlfors *et al.* have recently underlined the importance of B_f by showing that auditory brainstem response screening, a quantifiable method to evaluate bilirubin-induced neurotoxicity, correlates with B_f rather than with total bilirubin concentrations.[4] The protective role of HSA administration has also been investigated in neonates. Its efficacy, however, has never been established in randomized controlled trials. Two retrospective studies have shown reduced B_f concentrations in jaundiced neonates after HSA administration.[16,18] One additional small cohort study has shown some protective effect of HSA administration on the development of brain damage, as measured by auditory brainstem response screening.[17] Other studies, however, failed to demonstrate beneficial effects of HSA treatment.[15] Importantly, most human studies did not assess plasma B_f, or used methods that seriously underestimate B_f levels due to a 42-fold sample dilution.[15-18,29] The absence of reliable data on B_f concentrations obviously impeded the interpretability of these studies. In addition, human studies are intrinsically limited by the impossibility of measuring brain bilirubin levels. Recently, Ahlfors *et al*. automated and improved the available B_f test, while Zelenka *et al*. developed a sensitive method for tissue bilirubin determinations.[13,25] These newly developed methods allowed us to reliably measure B_f and brain bilirubin levels in two well-established animal models.[19,20]

We first investigated the efficacy of adjunct HSA treatment in moderately chronic hyperbilirubinemic Gunn rats. Routine phototherapy decreased unconjugated hyperbilirubinemia in these animals, while HSA alone increased plasma UCB levels. Routine phototherapy and HSA alone both decreased B_f and brain bilirubin levels. The decrease in brain bilirubin in the HSA-alone group was in concordance with previous animal studies by Diamond *et al.*, who described that bilirubin ^{14}C deposited in the brain could in part be mobilized and returned to the circulation by subsequent treatment with HSA.[2] Next we investigated adjunct HSA treatment during phototherapy in Gunn rats. Rats treated with adjunct HSA treatment had lower B_f and, to a lesser extend, brain bilirubin

levels, compared with phototherapy alone. These results, when taken together, support a model in which only B_f is able to move between the vascular and extravascular (tissue) compartment of the bilirubin pool (Figure 7A). This translocation of B_f occurs across both the vascular endothelial cells and the BBB. In this model, HSA administration could act *in tandem* with phototherapy. HSA first binds to plasma B_f, which decreases the B_f concentration within the vascular compartment. This decrease promotes a bilirubin shift from the extravascular pool, as reflected by the increased plasma UCB concentrations during HSA-treatment. The newly recruited intravascular bilirubin is then, after its exposure to photo-isomerization, rapidly transported to the liver and excreted *via* the bile (Figure 7B).

Our results in acutely jaundiced Gunn rats showed that APHZ administration induced a comparable hemolysis in all groups. Routine phototherapy thus did not affect the severity of hemolysis, or did treatment with HSA alone, as indicated by similar decreases in Hb and Ht (Figure 3). APHZ increased plasma UCB and B_f concentrations by 30–60% within two days after administration. Phototherapy mitigated this increase, but to a relatively small extent. HSA alone treatment again tended to increase, rather than decrease, plasma UCB concentrations. The most striking finding, however, was the synergistic effect of combined phototherapy and HSA treatment. Adjunct HSA not only decreased B_f concentrations in the plasma, but it also completely prevented the hemolysis-induced deposition of bilirubin in the brain, in contrast to phototherapy and HSA alone treatment.

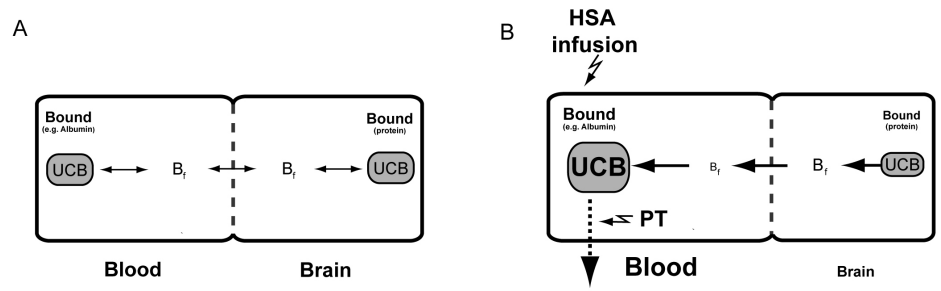

Figure 7. Human serum albumin (HSA) treatment during unconjugated hyperbilirubinemia.
A. Unconjugated hyperbilirubinemia may result in the accumulation of unconjugated bilirubin (UCB) within the brain. Only non-protein bound UCB (B_f) is able to move between the blood (*e.g.* vascular compartment) and the brain (*e.g.* extravascular compartment). **B**. Treatment with HSA decreases B_f levels within the blood. This promotes an B_f-induced shift of bilirubin from the brain into the circulation. Additional phototherapy (an essential step) subsequently converts this bilirubin into photo-isomers that can readily be excreted *via* the bile (dashed arrow).

The failure of HAS alone treatment demonstrates the importance of phototherapy in our model. When HSA induces a bilirubin shift from the extravascular to the vascular compartment, phototherapy is needed to convert this newly recruited intravascular bilirubin into photo-isomers that can be readily excreted *via* the bile. Without phototherapy, bilirubin will move back from the blood into the tissues as the plasma albumin levels return to baseline (*i.e.* within 48h; Figure

3D). The observed lack of effect during phototherapy alone on brain bilirubin levels may be time-related: phototherapy decreased plasma UCB within 36h, but did not decrease B_f levels until after 48h of treatment. Indeed, long-term phototherapy apparently circumvented this delayed decrease in B_f levels, and decreased both B_f and brain bilirubin in permanently jaundiced Gunn rats. We cannot exclude the possibility that non-protein bound bilirubin is less readily converted into photo-isomers than the protein bound fraction. Taken together, our results not only demonstrate the benefits of adjunct HSA, but also question the efficacy of phototherapy during acute hemolytic jaundice.

The correlation between plasma and brain bilirubin levels was virtually absent in our chronic and acute experiments. These data are consistent with clinical evidence that shows a poor predictive value of plasma bilirubin, especially above 300 μmol/L, for neurotoxicity.[6] Together, these observations illustrate that UCB is, at best, a poor predictor for bilirubin deposition within the brain. B_f concentrations correlated reasonably well with individual brain bilirubin levels in our experiments. Yet, the R^2-value in our acute experiment indicated that the variation in brain bilirubin is clearly not solely related to plasma B_f concentrations. Also, it is interesting to note that the HSA-induced decrease in brain bilirubin concentrations is less pronounced than the HSA-induced decrease in plasma B_f concentrations. These observations confirm that, apart from B_f, other factors (*e.g.* changes in blood pH, BBB integrity, active transport of bilirubin across the BBB, hemolysis, and inflammation) are also highly important in the pathogenesis of bilirubin-induced neurological damage.[30,31] It would be interesting to investigate these factors, as well as the accumulation of bilirubin in *specific* brain regions (since bilirubin predominantly accumulates in the deep nuclei of the brain) during HSA treatment in future animal experiments. Also, studies with different HSA dosages would be required to determine dose dependency relationships between HSA and its bilirubin effects, since it seems reasonable to assume that another dosage of HSA would result in quantitatively different outcomes. Taken together, these issues demonstrated the need for a further evaluation of HSA administration in future experiments.

It is worth noticing the differences between chronic and acute hyperbilirubinemia models. The acute model, in contrast to the chronic model, does not reflect a steady state condition. In the chronic model, the UCB production rate is stable, whereas the UCB production rate is increased in the acute model. This results in different kinetics that might influence the (re)distribution of bilirubin from the blood into the tissue compartment, and *vice versa*. To exclude the possibility that the differences between our models were induced by APHZ, rather than by hemolysis, we performed additional experiments. In these experiments we induced hyperbilirubinemia in Gunn rats *via* a different strategy, namely transfusion with 1-week old donor rat erythrocytes (data not shown). Rats were then treated with or without phototherapy. Compared with the APHZ results, the effects on total plasma UCB, B_f, brain bilirubin levels, and their interrelationships were similar. These results strongly indicated that the differences between our models were induced by hemolysis, and not directly by the APHZ compound.

For the interpretation and extrapolation of the results, we underline that species differences in bilirubin kinetics do apply between humans and rats, even when both are completely deficient in UGT1A1 activity (Crigler-Najjar type I patients and Gunn rats, respectively). For example, the hyperbilirubinemia in Gunn rats is less severe than that in Crigler-Najjar type I patients, and the natural course of the disease is milder. Furthermore, in Gunn rats the accumulation of bilirubin does not usually produce neonatal morbidity or a kernicterus pattern. Also, we studied adult Gunn rats because it was not feasible to reliably administer and assess the effects of phototherapy for 16 days in Gunn rat pups. The central nervous system is less vulnerable in adult rats. We are consequently aware that bilirubin distribution and affinities could be different in the neonatal or adult central nervous system.[32-34] Although the adult Gunn rat model has been proven valuable in studying bilirubin (patho)physiology, these observations justify some caution in extrapolating our results to Gunn rat pups or hyperbilirubinemic patients.

In our study we have used a commercially available HSA solution and found clear proof that it enhanced the therapeutic efficacy of routine phototherapy. We used human serum albumin (HSA) rather than rat serum albumin (RSA), to mimic the clinical situation as closely as possible, and to use a treatment that is presently already available for patients. The albumin solution used in our experiments is currently widely applied in neonates, which greatly increases its therapeutic potential, and will facilitate the setup of future clinical trials.[15-18,29] These trials should ideally incorporate B_f measurements and auditory brainstem response screening to monitor the efficacy of treatment. B_f measurements should be performed according to the recently developed method of Ahlfors *et al.*, that enables an automated and reliable measurement of B_f in a clinical setting. HSA administration has previously been used in jaundiced neonates, mainly before phototherapy became available. Although generally safe, HSA was associated with side effects, such as fluid overload. Theoretically, HSA administration could also induce infections or immunological reactions. The occurrence of these side effects, although uncommon, should be monitored in future clinical trials.[15-18,29]

Taken together, our data show that HSA enhances the efficacy of routine phototherapy in phototherapy-treated Gunn rats, both during permanent and acute jaundice. Our study underlines the need to critically evaluate the use of HSA as adjunct to phototherapy in randomized controlled clinical trials. We expect that a focus on tissue, rather than on plasma bilirubin concentrations, could induce a paradigm shift that will allow the development of increasingly efficient treatment strategies. These strategies will, hopefully, further decrease the burden of bilirubin-induced brain damage in the near future.

Acknowledgment

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Chapter 3

OPTIMIZING EXCHANGE TRANSFUSION FOR SEVERE UNCONJUGATED HYPERBILIRUBINEMIA: STUDIES IN THE GUNN

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ABSTRACT

Background: Severe unconjugated hyperbilirubinemia carries the risk of neurotoxicity. Phototherapy (PT) and exchange transfusion (ET) are cornerstones in the treatment of unconjugated hyperbilirubinemia. Studies to improve ET efficacy have been hampered by the low application of ET in humans and by the lack of an *in vivo* model. The absence of an appropriate animal model has also prevented the determination of the efficacy of adjunct or alternative treatment options such as albumin (Alb) administration.

Aim: To establish an *in vivo* model for ET, and to determine the most effective treatment (combination) of ET, PT and Alb administration.

Methods: Gunn rats received either PT, PT+Alb, ET, ET+PT, ET+PT+Alb or sham operation (each n=7). ET was performed *via* the right jugular vein in ~20 min. PT (18 μW/cm^2/nm) was started after ET or at T_0. Albumin *i.p.* injections (2.5 g/kg) were given after ET or before starting PT. Plasma unconjugated bilirubin (UCB), plasma free bilirubin (B_f), and brain bilirubin concentrations were determined.

Results: We performed ET in 21 Gunn rats with 100% survival. At T_1, ET was profoundly more effective in decreasing both UCB (-44%; $p<0.01$) and B_f (-81%; $p<0.05$) than either PT or PT+Alb. After 48h, the combination of ET+PT+Alb showed the strongest hypobilirubinemic effect (-54% compared with ET).

Conclusions: We optimized ET for severe unconjugated hyperbilirubinemia in the Gunn rat model. Our data indicate that ET is the most effective treatment option, in as well the acute as the follow-up situation.

INTRODUCTION

Neonatal jaundice carries the risk of neurotoxicity, due to the deposition of unconjugated bilirubin (UCB) in the central nervous system. Most of the UCB (~99%) in plasma is bound to plasma proteins (mainly albumin). Only a small fraction (~1%) is "free", and only this free bilirubin (B_f) has the ability to cross the blood-brain barrier and induces brain damage.[1-5]

Presently, the standard treatment for hyperbilirubinemia is phototherapy (PT). Phototherapy is generally effective, but in some neonates the plasma bilirubin concentrations become dangerously high or rise rapidly despite PT. In these patients PT might fail to prevent bilirubin-induced brain damage, and for these patients exchange transfusion (ET) is indicated. Exchange transfusions have more serious side effects and complications than PT. The mortality rate from the procedure is approximately 0.3-2.0%. Significant morbidity is associated with 5-12% of ETs.[6-8] Complications include cardiac arrest, thrombosis of the portal vein, graft vs. host disease, coagulopathies, hypoglycemia, hypocalcaemia, necrotizing enterocolitis, and transmission of infectious diseases.[6-10]

It has remained unclear whether ET could successfully be replaced by other, more effective treatment options. For example, albumin administration might be a good treatment modality. Recently, we found that adjunct human serum albumin (HSA) increased the efficacy of PT; it decreased plasma B_f concentrations and brain bilirubin levels by ~90% and ~70%, respectively.[11]

Studies to replace ET, to improve ET efficacy and/or to minimize its risks have been hampered by the contemporary low application rate of ET in humans, and by the lack of an appropriate *in vivo* model system. In order to better study the effects of an ET, animal studies would be highly desirable. An appropriate animal model should resemble the human situation as much as possible. In case of ET for hyperbilirubinemia, it should lower the bilirubin levels sufficiently, quickly, and safely. In this study we set out to establish an animal model for ET, in which we would be able to evaluate the effect of an ET on bilirubin concentrations in the acute and long-term situation.

We used Gunn rats suffering from hyperbilirubinemia due to a mutation in uridine-diphosphoglucuronosyltransferase: UGT1A1.[12-15] The Gunn rat is a well-established animal model for unconjugated hyperbilirubinemia. The histopathological lesions in severely kernicteric Gunn rats include damage to central auditory structures, especially the cochlear nuclei and inferior colliculi, and are similar to those found in human neonates with classic kernicterus.[16]

In the present study, we successfully optimized and verified an ET model in Gunn rats to compare acute treatments for severe hyperbilirubinemia. Next, we evaluated different acute treatment options for hyperbilirubinemia with or without the combination of ET, and compared total serum bilirubin, free bilirubin, and brain bilirubin levels.

ANIMALS, MATERIALS, AND METHODS

Animals

Homozygous male Gunn rats (RHA/jj; 10-12 weeks of age, bodyweight: 254-335 g) from our breeding colony were kept in an environmentally controlled facility, were fed *ad libitum*, and had free access to water. The Animal Ethics Committee of the University of Groningen (Groningen, The Netherlands) approved all experimental protocols.

Materials

Diet

Hope Farms B.V. (Woerden, The Netherlands) produced the semi-synthetic control diet (code 4063.02). This diet contained 13 energy% fat and 5.2 wt% long-chain fatty acids. Gunn rats were fed this diet during a 5-week run-in period, and during the experimental period.

Chemicals

Horseradish peroxidase type 1, D-glucose, glucose oxidase, and hydrogen peroxide were purchased from Sigma Chemical Co. (St. Louis, MO). Human serum albumin (Albuman®; 200 g/L, fatty acid free) was purchased from Sanquin (Amsterdam, The Netherlands).

Methods

Phototherapy

Two phototherapy devices were developed according to the prototype that was designed by Ostrow *et al.* and previously successfully used.[11,17] Each device consisted of two blue phototherapy lamps (Philips, TL-20W/52) suspended in a reflective canopy 30 cm above the bottom of the cage. Phototherapy (18 μW/cm^2/nm; 380-480 nm; measured by an Elvos-LM-1010 Lux meter at 30 cm distance), was administered continuously to Gunn rats, shaven on their back and flanks.

Exchange transfusion

Fresh whole rat Wistar donor blood was obtained from Harlan Laboratories B.V. (Horst, The Netherlands). Exchange transfusion was carried out under general anesthesia with isoflurane. Body temperature was maintained at 37-38 ºC by a heating plate. Saturation was checked and kept constant during the whole procedure above 95%. Different vessel approaches, including femoral artery and vein, and carotid artery and jugular vein, have been tested. The following description was used for all experiments. A small incision was made in the right throat region and, with the aid of an operating microscope, the right jugular vein was cannulated with heparinized silastic tubing for the extraction of the native blood, and the infusion of donor blood. In total 20 ml of native blood was taken out, and 20 ml of donor blood was infused *via* a heparinized lock (1 ml per cycle in 1 minute). Exchange transfusion was performed at a rate of 1 ml/min, for 20 minutes. Blood

outflow was performed by hand using 1 ml syringes, and donor blood inflow was performed using an infusion pump. After the exchange transfusion, tubes were ligated and left in situ, and the skin was sutured.

Sham transfusion

Sham transfusion was carried out following the same procedure as the exchange transfusion. After cannulation of the jugular vein, animals were kept under general anesthesia for 20 minutes. After the sham, the heparinized silastic tubings were ligated extra corporally and the proximal part was left in the jugular vein *in situ*. Finally, the skin was sutured.

Study design

Adult Gunn rats were randomized to receive either sham operation without treatment (controls), phototherapy, phototherapy+albumin (Alb), exchange transfusion, exchange transfusion+phototherapy, or exchange transfusion+Alb+phototherapy (each of these groups; n=7). The exchange transfusion (ET) group underwent ET at a rate of 1 ml/min for 20 minutes. Albumin *i.p.* injection (2.5 g/kg) was given immediately after the ET or right before PT was started. Heparinized samples of tail vein blood were collected under isoflurane anesthesia, at time (t)=0 (before the ET), at t=1, t=3, t=6, and t=24h after the ET. After 48h, all animals were exsanguinated *via* the descending aorta, and flushed *via* the same port with 100-150 ml NaCl 0.9% under isoflurane anesthesia. Brains were subsequently collected for the determination of tissue bilirubin levels. These samples were rinsed twice with phosphate buffered saline, snap frozen in liquid nitrogen, and immediately stored (wrapped in aluminum foil) at -80 °C until analysis.

Analytical Methods

Plasma analysis

Blood samples were protected from light, stored at -20 °C under argon directly after collection, and processed within 2 weeks. UCB and B_f were determined using a Zone Fluidics system (Global Flopro, Global Fia Inc, WA), as previously described by Ahlfors *et al.*.[18]

Tissue bilirubin analysis

Tissue bilirubin content was determined using HPLC with diode array detector (Agilent, Santa Clara, CA) as described earlier.[19] Briefly, 300 pmol of mesobilirubin in DMSO (used as an internal standard) was added, and samples were homogenized with glass dust using glass rod. Bile pigments were then extracted into chloroform/methanol/hexane (10:5:1) solution at pH 6.0, and subsequently extracted in a minimum volume of methanol/carbonate buffer (pH 10.0) to remove contaminants. The resulting polar droplet (extract) was loaded onto C-8 reverse phase column (Phenomenex, Torrance, CA) and separated pigments were detected at 440 nm. The concentration of bilirubin was calculated as nmol/g of wet tissue weight. All steps were performed under dim

light in aluminum-wrapped tubes. We did not specifically measure bilirubin deposition in the brain nuclei, but relied on total tissue bilirubin measurements.

Statistical analysis

Normally distributed data that displayed homogeneity of variance (by calculation of Levene's statistic) were expressed as mean ± SD, and analyzed with parametric statistical tests. Analysis of variance (ANOVA) with post-hoc Tukey correction was performed for comparisons between groups, and the Student *t* test for comparison of paired data within groups. The level of significance was set at $p<0.05$. Analyses were performed using SPSS Statistics 20.0 for Windows (SPSS Inc., Chicago, IL).

RESULTS

Development and validation of the model

In the human situation the common route for ET involves catheterization of the umbilical vein, and arteriovenous or venovenous exchange. Initially, we set out to establish arteriovenous exchange *via* the femoral artery and vein. Based on the anatomical location, the femoral artery was not suitable for the exchange procedure. We then switched first to arteriovenous exchange *via* the carotid artery and jugular vein, but this method failed because of the high pressure in the carotid artery, which made it impossible to keep the cannula in place for more than 5 minutes. Finally, we moved on to venovenous exchange *via* the jugular vein on both sides. When we found out that we received the same results in decrease of plasma bilirubin concentrations *via* the push-and-pull-method *via* one jugular vein, as *via* the continuous exchange *via* both jugular veins, we decided to continue with the venovenous exchange *via* one jugular vein. The rats recovered quickly with this method. *Via* the same jugular vein we extracted the blood of the Gunn rat, and infused fresh Wistar donor blood (UCB <1 mg/dL), in 20 minutes. We tested different lengths of the procedure, and observed that the 20 minutes procedure led to the same decrease of plasma UCB concentrations as the 40 and 60 minutes procedures (same volume, data not shown).

We performed an ET in 21 Gunn rats with 100% survival. The recovery after ET was rapid, illustrated by maintenance of body weight during the 48h after ET (at T_{48}; 100 ± 3% compared with T_0; NS). Figure 1A shows the course of plasma UCB concentrations after ET. ET rapidly decreased plasma UCB concentrations from 14.9 mg/dL at T_0, to 8.3 mg/dL at T_1 (-44%; $p<0.001$). In Figure 1B the course of plasma B_f concentrations after ET is shown. ET decreased plasma B_f concentrations from 11.1 µg/dL at T_0, to 2.1 µg/dL at T_1 (-81%; $p<0.001$). At T_6, T_{24}, and T_{48} no significant differences existed in plasma B_f concentrations between controls and ET.

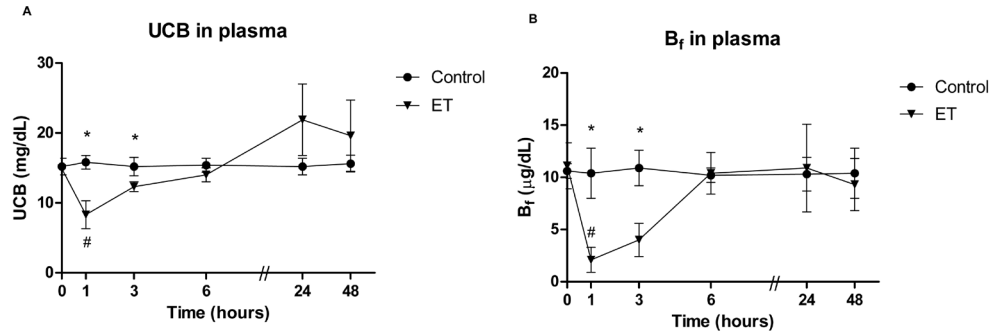

Figure 1. Course of plasma UCB and B_f concentrations after exchange transfusion.
Course of plasma UCB concentrations (**A**) and plasma B_f concentrations (**B**) after sham transfusions (control) or an exchange transfusion (ET) in Gunn rats. Rats were randomized to receive sham transfusions (control) or an exchange transfusion. Values are mean ± SD. *$p<0.01$ compared with controls. #$p<0.001$ T_0 compared with T_1 in ET-group.

Plasma UCB concentrations after 1h

We compared the acute effect of the different treatments; PT, ET, Alb administration or a combination thereof (Figure 2A). After 1h, PT showed no significant differences in plasma UCB concentrations compared with controls. PT+Alb showed a significant increase compared with controls ($p<0.001$). In contrast, ET reduced plasma UCB concentrations by 47% within 1h ($p<0.001$ vs controls). The addition of either PT or the combination of PT and Alb did not significantly augment this hypobilirubinemic effect. Each of the combination therapies that included ET resulted in a significantly lower plasma UCB concentration compared with the control, PT or PT+Alb groups (each $p<0.001$).

Plasma B_f concentrations after 1h

Figure 2B shows the effects of the different treatment combinations on plasma B_f concentrations. After 1h, PT and PT+Alb reduced plasma B_f concentrations with 35% ($p<0.001$ vs controls) and 53% ($p<0.001$ vs controls), respectively. For the ET-group, ET+PT-group, and ET+PT+Alb-group, the decrease in plasma B_f concentrations was even more profound (-80%, -80%, and -89%, respectively; each $p<0.001$ vs controls, no statistically significant differences between the three ET-groups). Also, the different ET-groups each showed significantly lower plasma B_f concentrations compared with PT+Alb ($p<0.05$).

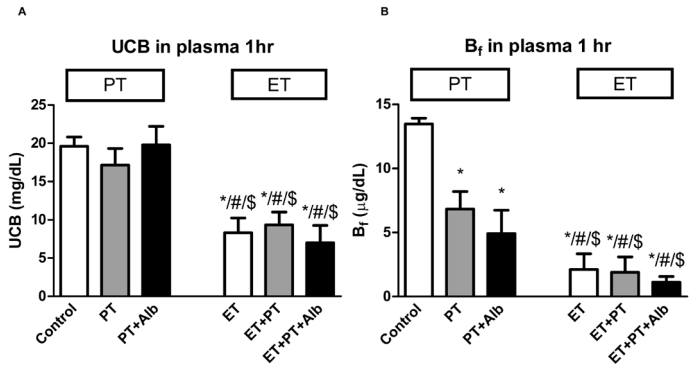

Figure 2. Plasma UCB and B_f concentrations after 1h.
Acute effects of sham transfusions (control), phototherapy (PT), albumin (Alb), an exchange transfusion (ET), or a combination of these on plasma UCB concentrations (**A**) and plasma B_f concentrations (**B**) in Gunn rats. Rats were randomized to receive sham transfusions (control) or an exchange transfusion, and were subsequently treated with phototherapy, albumin, or the combination of phototherapy+albumin. Values are mean ± SD. *p<0.001 compared with controls. #p<0.05 compared with PT. $p<0.05 compared with PT+Alb.

Plasma UCB concentrations after 48h

We also determined the long-term (48h) hypobilirubinemic effect of the different treatments. We compared treatment combinations that are also used in clinical practice: an ET with or without the combination of PT and/or Alb administration. Figure 3A shows the effects of the different treatment combinations on plasma UCB concentrations after 48h. In the ET-group the plasma UCB concentrations returned back to physiological Gunn rat values as described in the validation of the model. ET+PT significantly reduced plasma UCB concentrations compared with ET after 48h (-36%; p<0.05). Albumin further potentiated this effect, shown in the significant decrease of ET+PT+Alb compared with ET+PT (-28%; p<0.05).

Plasma B_f concentrations after 48h

Figure 3B shows the effects of the different treatment combinations on plasma B_f concentrations. After 48h, the plasma B_f concentrations of the ET-group was still significantly lower compared with controls (-48%; p<0.05 vs controls; data not shown). ET+PT further reduced plasma B_f concentrations with 47% (NS; vs ET). Albumin potentiated the decrease in plasma B_f concentrations after 48h, shown in a profound, significant decrease of ET+PT+Alb compared with ET (-81%; p<0.01).

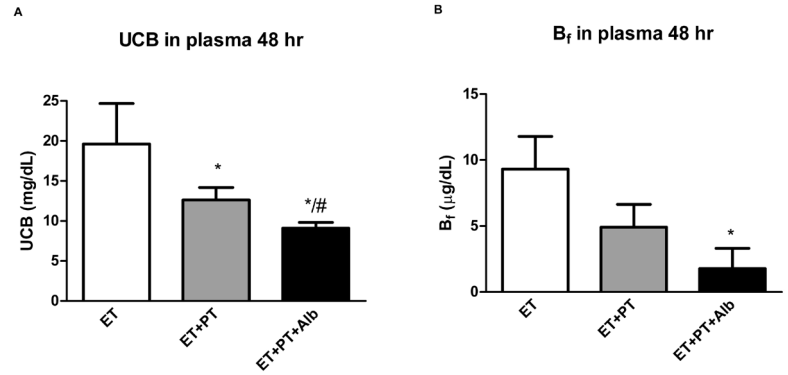

Figure 3. Plasma UCB and B_f concentrations after 48h.
Long-term effects of an exchange transfusion (ET), with or without the combination of phototherapy (PT), and/or albumin (Alb), on plasma UCB concentrations (**A**) and plasma B_f concentrations (**B**) in Gunn rats. Rats were randomized to receive an exchange transfusion, and were subsequently treated with phototherapy, albumin, or the combination of phototherapy+albumin. Values are mean ± SD. *p<0.01 compared with ET. #p<0.05 compared with ET+PT.

Brain UCB levels

Figure 4 shows that PT+Alb decreased brain bilirubin levels by 63% (p<0.001), compared with untreated controls. Adjunct Alb thus lowered brain bilirubin levels by an additional 33% (NS), compared with PT alone. ET+PT+Alb decreased brain bilirubin levels by 61% (p<0.01), compared with ET. Adjunct Alb thus lowered brain bilirubin levels by an additional 57% (p<0.01), compared with ET+PT.

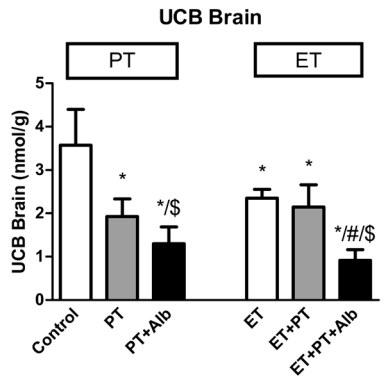

Figure 4. Brain bilirubin levels.
Effects of sham transfusion (controls), phototherapy (PT), albumin (Alb), an exchange transfusion (ET), or a combination of these on brain bilirubin levels in Gunn rats. For experimental setup, we kindly refer to the Methods section. Values are mean ± SD. *p<0.05 compared with controls. #p<0.01 compared with PT. $p<0.05 compared with ET and ET+PT.

DISCUSSION

In this study we successfully optimized ET during unconjugated hyperbilirubinemia in a Gunn rat model. We also bring the evidence that this Gunn rat-ET model might be very valuable to evaluate the effect of modulating ET procedures and techniques, and to compare its efficacy in combination with other treatments to prevent brain damage during acute severe hyperbilirubinemia. Our data indicate that ET is highly effective in decreasing UCB and B_f within 1h of treatment, and that combining ET with either PT or PT+Alb does not further significantly potentiate this rapid hypobilirubinemic effect. As follow-up treatment after ET, the combination of PT with Alb is most effective in maintaining this hypobilirubinemic effect over 48h.

Presently, ET is a very effective alternative treatment to PT in severely jaundiced neonates. An ET is considered as a "rescue treatment", if plasma UCB levels are severely elevated or fail to respond to PT. Exchange transfusion generally reduces plasma UCB concentrations by 50%, although the efficacy varies with the severity of the ongoing hemolysis, and the amount of bilirubin that re-enters the circulation from the tissues.[20] This re-entry occurs due to the diffusion of B_f from the tissue pool into the plasma pool, and decreases the risk of bilirubin-induced neurotoxicity.[20] Eventually, all therapies should aim to prevent neurotoxicity, and this can only be achieved by decreasing (brain) tissue UCB levels rather than plasma UCB concentrations. Nevertheless, an ET has a considerable morbidity, and even mortality has been reported.[6-8] Fortunately, the need for ETs has been greatly reduced since the introduction of PT.[21,22]

Our model makes it possible to determine if we can replace ET, improve its efficacy and/or minimize its risks. An alternative treatment option would be the administration of human serum albumin (HSA). HSA administration can be used in combination with an ET in severely jaundiced neonates, when donor blood is not immediately available[23], but this approach has been disputed.[23,24] The rationale for HSA administration is that the resultant increase in albumin concentration will enhance the bilirubin/albumin-binding capacity in the intravascular compartment, thereby promoting the mobilization of bilirubin from extravascular tissues, including the central nervous system, into the circulation. In this way, albumin is used as an adjunct treatment in order to more efficiently remove bilirubin.[23]
Our experimental design on albumin administration differs from clinical ET practices in humans with respect to dosage, timing, and route of administration. In the clinics, albumin may be administered to hyperbilirubinemic neonates, but its use seems relatively rare. If administered, it has been advised to do so prior to ET, aimed to increasing its efficacy by mobilizing bilirubin from tissues. In the present study, we administered albumin immediately after ET, aimed at preventing or mitigating a possible rebound of B_f after ET. We administered albumin in a relatively high dosage (2.5 g/kg, rather than ~1 g/kg in humans) *via i.p.* bolus injection, in contrast to *i.v.*

administration in humans. It should be underlined that these methodological differences prevent direct extrapolation of our present result towards the clinical situation. Rather, present positive "proof of principle" results in our rat model support the design of clinical studies in this direction.

Recently, we found that HSA enhances the efficacy of routine PT in phototherapy-treated Gunn rats, both during permanent and acute jaundice.[11] We speculated that HSA and PT work *in tandem*: HSA binds bilirubin within the plasma, and PT then promotes its excretion *via* the bile.[11] In this study we showed that albumin administration in combination with either PT or ET is already effective after 1h of treatment. Furthermore, we showed that the combination of PT+Alb is effective in decreasing plasma UCB concentrations, plasma B_f concentrations, and brain UCB levels after 48h. However, in this study we found that ET decreases UCB and B_f concentrations even more than PT+Alb, both in the acute and the chronic treatment situation. Our data demonstrate that ET is still the most effective treatment option in acute severe hyperbilirubinemia. Unfortunately, we were not able to measure plasma albumin concentrations. Albumin is believed to exert its beneficial effects in the blood circulation. In a previous study in adult Gunn rats, we showed that HSA readily enters the plasma compartment after *i.p.* injections.[11] It is worth mentioning that in this previous study we have applied "albumin only" treatment, *i.e.* without prior ET. "Albumin only" decreased the plasma B_f with 54% after 48h.[11] The present results justify a follow-up study on an ET+Alb-group. However, in the present study design we focused on the comparability of our animal-experiments with the clinical situation. Next, it is worth mentioning that in adult rats, PT may not be as efficient as in rat pups. Both skin thickness and body mass/surface ratio are increased in adults, thus underestimating the potential of PT together with ET.

In our ET-model we chose a venovenous exchange *via* one jugular vein in 20 minutes. Various models for exchange of blood are described, each for different purposes. Eguchi *et al.* performed a total blood exchange (TBE) in rats, and showed that TBE suppressed the early stage of liver regeneration following partial hepatectomy.[25] These scientists performed the TBE *via* the right femoral vein and artery. Henry *et al.* improved monoclonal antibody tumor/background ratios with ETs in rats.[26] They used the right common carotid artery for the blood exchange. Takeda *et al.* studied the effect of blood ET as an initial treatment of acute hemorrhagic pancreatitis in rats.[27] Blood ET was performed *via* a previously indwelt tube in the inferior vena cava. Hodges *et al.* studied the effect of an ET on the efficacy of penicillin therapy of pneumococcal infection in rats.[28] The left external jugular vein was used to perform the ET. We based our model to a certain extent on the latter approach. Kurantsin-Mills *et al.* studied flow dynamics of human sickle erythrocytes in the mesenteric microcirculation of rats that underwent an ET via the femoral vein.[29] The time schedule we used for blood outflow and infusion was partly based on this study. Since we had a different goal than the studies described above, namely exchange of hyperbilirubinemic blood, we decided to develop our own model.

We used fresh donor-rat blood, collected at the same day as the ET took place. The life span of erythrocytes is approximately 120 days in human adults, 90 days in neonates, and 50-60 days in rats.[30] Therefore, the storage time of red blood cells for rats is much shorter than for human red blood cells, maximum 7 days compared with maximum 30 days, respectively.[31] Hemolysis of rat red blood cells happens quickly, and after 7 days all the red blood cells are lysed.[31]

In conclusion, we successfully optimized and verified an animal model for ET for treatment of severe unconjugated hyperbilirubinemia. Our data indicate that ET is a more effective treatment option for acute hyperbilirubinemia, than either PT or the combination of PT+Alb. The combination of PT+Alb was the most effective follow-up treatment after ET for a long term (48h) hypobilirubinemic effect. The availability of this optimized model could be very helpful to further optimize the treatment for acute, potentially neurotoxic hyperbilirubinemia.

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Chapter 4

ALBUMIN ADMINISTRATION PROTECTS AGAINST BILIRUBIN-INDUCED AUDITORY BRAINSTEM DYSFUNCTION IN GUNN RAT PUPS

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ABSTRACT

Background: Free bilirubin (B_f), the unbound fraction of unconjugated bilirubin (UCB), can induce neurotoxicity, including impairment of the auditory system, which can be assessed by brainstem auditory evoked potentials (BAEPs). We hypothesized that albumin might reduce the risk of neurotoxicity by decreasing B_f and its translocation into the brain.

Aim: To determine the effects of albumin on BAEPs and brain bilirubin content in two Gunn rat pup models of acute hyperbilirubinemia.

Methods: We used Gunn rat pups, which have a deficiency of the bilirubin-conjugating enzyme UGT1A1. We induced hemolysis by injection of phenylhydrazine (phz) into 14-days old pups. Subsequently, pups were treated with either *i.p.* human serum albumin (HSA; 2.5 g/kg; n=8) or saline (control; n=8). We induced acute neurotoxicity by injecting 16-days old pups with sulfadimethoxine (sulfa) and treated them with either HSA (n=9) or saline (control; n=10). To assess bilirubin-neurotoxicity we used the validated BAEP method and compared relevant parameters: *i.e.* peak latency values and interwave-interval (IWI) between peak I and peak II, a marker of acute neurotoxicity.

Results: Phenylhydrazine and sulfa significantly increased IWI I-II by 26% and 29% ($p< 0.05$) in the hemolysis model and the displacement model, respectively. Albumin completely prevented the increase of IWI I-II in either model. The beneficial effect of albumin in the displacement-model by means of normal BAEPs was in line with less bilirubin in the brain (NS). Interestingly, in the hemolysis-model the accumulation of total bilirubin in the brain was unaltered, and BAEPs still appeared normal. This might advocate for a role of brain B_f which was calculated, and showed that albumin treatment non-significantly reduces B_f concentrations in brain, compared to saline treatment.

Conclusions: Albumin treatment is neuroprotective in acute hyperbilirubinemia in Gunn rat pups. Our present results underline the importance of functional diagnostic test of neurotoxicity above biochemical concentrations.

INTRODUCTION

Unconjugated hyperbilirubinemia is considered a physiological and transient phenomenon, which occurs in many newborn infants. In case of severe hyperbilirubinemia or in vulnerable preterm infants, potentially devastating neurological sequelae may occur due to the deposition of toxic unconjugated bilirubin (UCB) in the central nervous system (CNS).[1] This underlines the need for additional treatment strategies to current therapies, *i.e.* phototherapy and exchange transfusion. Only free bilirubin (B_f), the fraction of UCB not bound to plasma proteins (*e.g.* albumin), can induce neurotoxicity after translocation across the blood-brain barrier (BBB). A few studies have been performed on administration of albumin in animals as well as in neonates to reduce the risk of bilirubin neurotoxicity, but detailed mechanistic data are lacking.[2-6] We hypothesized that albumin can be neuroprotective by decreasing B_f, and thus preventing its translocation into the CNS.

Bilirubin-neurotoxicity can be assessed by brainstem auditory evoked potentials (BAEPs), based on the vulnerability of the auditory system to hyperbilirubinemia. BAEPs (or auditory brainstem responses, ABRs) assess neural transmission between the auditory nerve and auditory brainstem structures. Bilirubin-induced auditory dysfunction can present as sensorineural hearing loss or auditory processing abnormalities, *i.e.* auditory neuropathy spectrum disorders (presumably due to damage of brain stem structures).[7]

We tested our hypothesis in Gunn rats, the well-established animal model for hyperbilirubinemia. Gunn rats spontaneously develop jaundice due to a mutation in uridine diphosphoglucuronosyl-transferase: UGT1A1.[8-10] This mutation is homologous to human patients with Crigler-Najjar type I syndrome and analogous to the relative deficiency of UGT1A1 activity seen in human neonates during the first several days of life. The histopathological lesions in severely kernicteric Gunn rats include damage to central auditory structures, especially the cochlear nuclei and inferior colliculi, and are similar to those found in human neonates with classic kernicterus.[11] The j/j homozygous Gunn rat pups show reduced postnatal cerebellar weight, and upon treatment with sulfadimethoxine, acute signs of hyperbilirubinemic encephalopathy.

For this study we used two Gunn rat pup models of acute hyperbilirubinemia mimicking severe neonatal hyperbilirubinemia: one due to hemolysis, and the other one based on drug-induced displacement of bilirubin from albumin. For the hemolysis model we used phenylhydrazine (phz) to induce hemolysis. For the bilirubin-albumin displacement model we used sulfadimethoxine (sulfa), which is a compound that competes with bilirubin for binding to serum albumin and results in accumulation of bilirubin in lipophilic tissues, including the brain.[2,12-14]

In this study, we evaluated the possible beneficial effects of albumin treatment on BAEPs in a rat pup model of acute hyperbilirubinemia due to hemolysis or due to bilirubin-albumin displacement. We show that albumin treatment is neuroprotective in acute hyperbilirubinemia in Gunn rat pups, irrespective of its nature, *i.e.* induction by hemolysis or by bilirubin-albumin displacement. Present results favor the clinical potency of albumin treatment to prevent or mitigate neurotoxicity by acute hyperbilirubinemia.

ANIMALS, MATERIALS, AND METHODS

Animals

Homozygous Gunn rat pups (jj; 14-16 days old) from the Virginia Commonwealth University Gunn rat breeding colony were used. The Gunn rats were housed per litter and were kept in an environmentally controlled facility. The adult mother rats were fed chow *ad libitum* and had free access to water. All procedures were approved by the institutional animal care and use committee of the Virginia Commonwealth University.

Materials

Chemicals

Phenylhydrazine (phz), sulfadimethoxine (sulfa), human serum albumin (HSA), horseradish peroxidase type 1, D-glucose, glucose oxidase, and hydrogen peroxide were purchased from Sigma Chemical Co. (St. Louis, MO).

Methods

Study design

Initially a blood sample (50-85 µl) was drawn *via* a cheek puncture to assess hematocrit and total plasma unconjugated bilirubin concentrations with a Leica Unistat Bilirubinometer (Reichert, Inc., Depew, NY, USA). Based on our previous experience, we have determined that approximately 50% of jj rats with UCB less than 9.0 mg/dL exhibit BAEP abnormalities following sulfa treatment, whereas 85% of jj rats with UCB levels greater than 9.5 mg/dL exhibit BAEP abnormalities following sulfa treatment (unpublished observations). Animals with higher UCB concentrations (>13.5 mg/dL) tended to have higher mortality rates in longer studies. Thus, we refined our experiments to reduce the total number of animals required, by only using jj rats with UCB levels between 9.5 and 13.5 mg/dL in this study.

Hemolysis model

In this model, we induced hemolysis by injection of phz (50 mg/kg bodyweight) into 14-days old Gunn rat pups to mimic neonatal hyperbilirubinemia. After injection, rat pups were subsequently treated with either *i.p.* HSA (2.5 g/kg; n=8) or saline (control; n=8) 10 minutes and 24h after phz injection. At day 16 of age, BAEP-measurements were performed. Immediately after BAEP-measurements, all animals were exsanguinated *via* a heart puncture and flushed *via* the same port with 50-100 ml NaCl 0.9% under isoflurane anesthesia. The brain and liver were subsequently harvested for the determination of tissue bilirubin levels. These samples were rinsed 2 times in phosphate buffered saline, snap frozen in liquid nitrogen, and immediately stored (wrapped in aluminum foil) at -80 °C until analysis.[15]

Displacement model

In the bilirubin-albumin displacement model, we induced exacerbation of hyperbilirubinemia (*i.e.* acute neurotoxicity) by injecting 16-days old Gunn rat pups with sulfa (200 mg/kg bodyweight) and treated them with either HSA (2.5 g/kg; n=9) or saline (control; n=10) 10 minutes after sulfa injection. Three animals in the sulfa group (1 Sulfa/Alb, 2 Sulfa/Sal) did not survive. Four hours after sulfa injection the BAEP-measurements were performed. Immediately after BAEP-measurements, all animals were exsanguinated and brain and liver were subsequently harvested for the determination of tissue bilirubin levels, as described above.[15]

Brainstem auditory evoked potentials (BAEP) stimulus and recording

Brainstem auditory evoked potentials (BAEPs) are a very sensitive, non-invasive tool to evaluate auditory nerve and brainstem function. BAEPs are in fact surface recorded electroencephalogram (EEG) responses recorded for the first 10 ms following an auditory stimulus (click) and averaged following many stimuli. As BAEPs are averaged, the stimulus evoked responses of the ascending auditory nervous system are resolved from the background EEG which is random following the stimulus, and the resultant waveforms represent the responses of the auditory nerve, the cochlear nucleus and superior olivary complex to the click.

Briefly, animals were lightly anesthetized with acepromazine (4.5-6.0 mg/kg) and ketamine (45-60 mg/kg) *i.m.*. Supplemental anesthesia, one quarter to one half of the original dose, was administered as needed if muscle artifact became too prominent. BAEPs were recorded using a Nicolet Spirit 2000 Evoked Potential System (Biosys, Inc.). The left ear was occluded with petrolatum, to minimize stimulation of the contralateral ear, and BAEPs were obtained to monaural 100 µs duration rarefaction clicks delivered at 31.7/s to the right ear through a Sony Walkman 4LIS headphone speaker.[1,13,14,16] The sound intensity was nominally set at 70 dB, which corresponded to a level of about 62 dB above a normal jj Gunn rat pup BAEP threshold level.[16] Surface electrical activity was recorded from 13 mm long subcutaneous platinum needle electrodes inserted on the scalp over the vertex and behind the left and right mastoid bullae with a ground electrode in the flank. Rectal temperature was controlled at 37.0±0.1 °C using a controller and heat lamp with a red bulb. The animal's temperature was stabilized for a minimum of 5 minutes before recordings were initiated. Two channel BAEP recordings were obtained from the contralateral to the ipsilateral mastoid (horizontal) and the vertex to the ipsilateral mastoid (vertical) electrode pairs, and filtered from 30 to 3000 Hz. Only the horizontal data are presented; the vertical data were used to help identify uncertain peaks. All recordings were done in a sound-attenuated room. Each individual BAEP was the averaged response to at least 2000 stimuli, and three or more replicated responses were obtained for each animal. The individual BAEP replications were then added, and the peaks and following troughs were scored using a cursor. The latency of wave I is the time from the stimulus to the peak of wave I. Other stimulus to peak latency values were subtracted to obtain interwave-intervals between wave peaks to arrive at values for the I-II and II-III interwave-

intervals. Wave IV is much more variable and historically does not show consistent abnormalities in this model, thus the wave IV data are not presented.[7] To assess bilirubin neurotoxicity we used the validated BAEP method and compared relevant parameters between albumin treated and control rats pups (peak latency values and interwave-interval (IWI) between peak I and peak II). An increased IWI I-II is a reflection of acute neurotoxicity.[7]

Analytical Methods

Plasma UCB and B_f analysis

Blood samples were protected from light, stored at -20°C directly after collection and processed within 4 weeks. UCB and B_f were determined using a Zone Fluidics system (Global Flopro, Global Fia Inc, WA).[17] Since all bilirubin is unconjugated in jj Gunn rats, B_f equals the total free bilirubin concentration in these animals. The HRP reaction is based on the observation that HRP catalyzes the oxidation of B_f by peroxide but does not catalyze the oxidation of albumin-bound bilirubin.[18] This can be described by the following equation:

$$\text{AlbUCB} \overset{K}{\leftrightarrow} \text{Alb} + B_f + \text{HRP} \overset{Kp}{\leftrightarrow} \text{oxidized } B_f \quad [1]$$

in which AlbUCB is the total amount of bilirubin that is bound to albumin in the plasma (>99.9%), K is the affinity constant of albumin for B_f, Kp is the rate constant for the HRP-catalyzed peroxide oxidation of B_f, and oxidized B_f is the amount of B_f that is oxidized by HRP. The B_f can then be calculated from the change in AlbUCB absorbance over time, as measured in a spectrophotometric flowcell at A460 nm. This can be described as follows:

$$\frac{dAlbUCB}{dt} = Kp \cdot HRP \cdot B_f \quad [2]$$

in which only the B_f is unknown since HRP is known, Kp can be determined, and dAlbUCB/dt is measured. Clearly this calculation is only accurate if K >> Kp in [1]. Unfortunately, this condition is rarely completely met during B_f measurement in plasma. As a result, the B_f will decrease after addition of HRP, resulting in an underestimation of the actual B_f concentration.[19] This problem can be solved by using different HRP concentrations to correct for the rate limiting dissociation of bilirubin from albumin. The correct B_f can then be determined as the reciprocal of the y intercept of a plot of $1/B_f$ versus the corresponding HRP concentrations.[17]

Tissue UCB analysis

Tissue bilirubin content was determined using HPLC with diode array detector (Agilent, Santa Clara, CA, USA) as described earlier.[15] Briefly, 300 pmol of mesobilirubin in DMSO (used as an internal standard) was added and samples were homogenized on ice. Bile pigments were then extracted into chloroform/hexane (5:1) solution at pH 6.0, and subsequently extracted in a

minimum volume of methanol/carbonate buffer (pH 10.0) to remove contaminants. The resulting polar droplet (extract) was loaded onto C-8 reverse phase column (Phenomenex, Torrance, CA, USA) and separated pigments were detected at 440 nm. The concentration of bilirubin was calculated as nmol/g of wet tissue weight. All steps were performed under dim light in aluminum-wrapped tubes. We did not specifically measure bilirubin deposition in the brain nuclei, but relied on total tissue bilirubin measurements.

Brain B_f analysis

Brain free bilirubin content was determined using the methods used by Daood *et al.*.[20] Brain bilirubin content was determined as described above. Total brain albumin was determined using the methods used by Ericsson *et al.*.[21] Briefly, 100 mg of brain tissue was added to 2% sodium dodecyl sulfate (SDS) in PBS buffer, and samples were disintegrated by sonication on ice. Next, samples were incubated at 70°C and shaked at 1400 rpm for 10 minutes. Samples were diluted with PBS to a total amount of SDS 1%. Albumin was measured by ELISA kit for rat albumin (E91028Ra, USCN, TX, USA). All samples were measured twice by ELISA reader Sunrise (Tecan, Austria) at 450 nm.

Total brain bilirubin and brain albumin values together with the rat albumin-bilirubin binding constant permits the calculation of CNS B_f levels. More specifically, CNS B_f was calculated using the published *in vivo* albumin-bilirubin binding *k* mean (9.2 L/µmol) values from Gunn rat pups (16 ± 0.5 days old)[22] in the following equation.[20]

$$\frac{TBB - B_f}{Alb} = \frac{B_f \cdot k_1}{1 + (B_f \cdot k_1)} + \frac{B_f \cdot k_2}{1 + (B_f \cdot k_2)}$$

in which TBB is total brain bilirubin, B_f is the CNS unbound bilirubin fraction, Alb is albumin in brain and *k* is the binding constant. This equation asumes independent binding of UCB to two sites on albumin[23,24]: k_1 and k_2 are the binding constants for the first and the second sites, respectively, with k_1 given by the binding constant value defined above[22] and k_2 equal to $k_1/15$.[23,24]

Statistical Analysis

Physiological data (body weight, total plasma bilirubin, free bilirubin and hematocrit) between the 5 groups (described under hemolysis-model and displacement-model) were compared by separate one-way ANOVAs with Tukey post-hoc analyses. The BAEP latency data were analyzed with a repeated measures ANOVA to determine if there was a significant main effect. For parameters with a significant main effect, one-way ANOVAs were performed to determine group differences between the interwave-intervals followed by Tukey post-hoc analyses. Spearman's rank correlation coefficients (R) were calculated. The level of significance was set at p-value below 0.05. Analyses were performed using PASW Statistics 18.0 for Windows (SPSS Inc., Chicago, IL).

RESULTS

Baseline characteristics

The initial physiological parameters compared between groups were the baseline weight, total plasma bilirubin (UCB), and hematocrit (Hct) (Table 1). There were no significant differences in the baseline values of these parameters between any groups.

Table 1. Physiological parameters at baseline.

Physiological parameters at baseline				
Group (n)	Weight (g)	UCB (mg/dL)	Hct (L/L)	UCB change (%)
Sal/Sal (9)	37 ± 5	12.4 ± 1.0	34.1 ± 1.9	13.4 ± 3.1 (+8)
Phz/Sal (8)	33 ± 5	12.0 ± 0.7	35.6 ± 1.3	19.3 ± 2.7 (+61)
Phz/Alb (8)	36 ± 3	11.6 ± 1.4	35.3 ± 2.4	27.0 ± 1.7 (+133)
Sulfa/Sal (10)	35 ± 5	12.7 ± 1.1	32.1 ± 2.2	4.5 ± 1.0 (-65)
Sulfa/Alb (9)	35 ± 5	12.7 ± 1.1	33.0 ± 2.1	6.3 ± 1.7 (-50)
Values are mean ± SD				

Plasma UCB concentrations

In Figure 1 plasma UCB concentrations are shown. In the hemolysis model, the Phz/Sal-treated animals had significantly higher plasma UCB concentrations compared to controls (+44%; $p<0.05$). Interestingly, the Phz/Alb-treated animals had significantly higher plasma UCB concentrations compared to Phz/Sal-treated animals (+40%; $p<0.05$). The displacement-model showed lower UCB concentrations compared to the hemolysis-model (-77%; $p<0.05$). The Sulfa/Sal-treated animals had significantly lower plasma UCB concentrations compared to controls (-66%; $p<0.05$). As in the hemolysis model, the Sulfa/Alb-treated animals also showed higher plasma UCB concentrations compared to Sulfa/Sal-treated animals (+40%).

Brainstem Auditory Evoked Potentials

Representative BAEP waves of each treatment group are depicted in Figure 2. The vertical dashed lines provide a visual demonstration of the increased latency of waves II and III following phz (hemolysis) or sulfa (acute bilirubin toxicity) treatment. For statistical comparison the Sal/Sal-group was used as the control group to which all other groups were compared.

Phz and sulfa significantly increased the interwave-interval (IWI) I-II in both the hemolysis- and the displacement model (by +26%, and +29%, respectively; $p<0.05$). Albumin completely prevented the increase of IWI I-II in either model of acute hyperbilirubinemia (Figure 3).

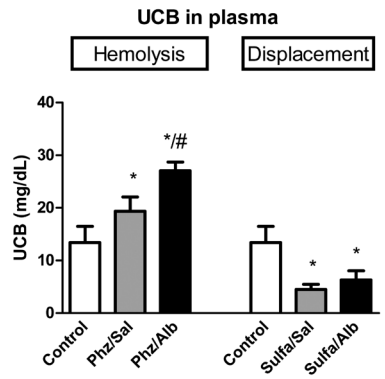

Figure 1. Plasma UCB concentrations.
Effects of no treatment (control) or albumin (Alb) on plasma UCB concentrations in as well the hemolysis (phz) as the displacement (sulfa) model for hyperbilirubinemia in 16-days old Gunn rat pups. Pups were randomized to receive saline (control), phenylhydrazine (phz) or sulfadimethoxine (sulfa), and were subsequently treated with saline or albumin (Alb). Values are mean ± SD. *$p<0.05$ compared to controls. #$p<0.05$ compared to Phz/Sal.

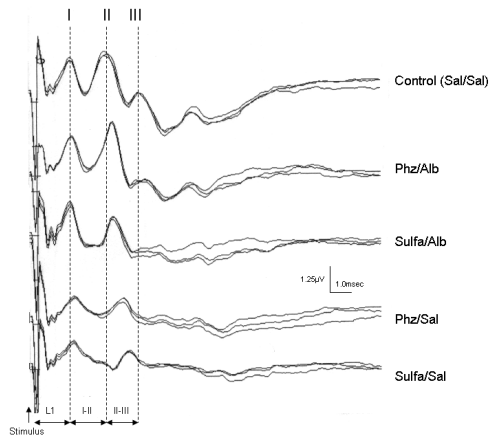

Figure 2. Representative BAEP waves.
Representative BAEP waves per treatment group. Representative BAEP waves from each of the treated Gunn rat pup groups: control (Sal/Sal), Phz/Alb, Sulfa/Alb, Phz/Sal, Sulfa/Sal. The vertical dashed lines provide a visual demonstration of the increased latency of waves II an d III following phenylhydrazine (hemolysis) or sulfadimethoxine (acute bilirubin toxicity) treatment

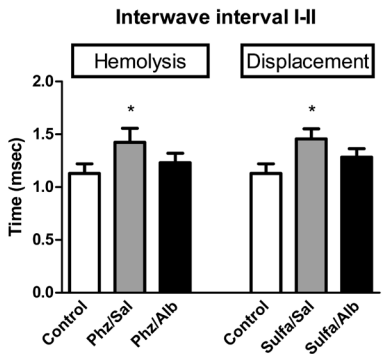

Figure 3. Quantitation of BAEP parameters.
Quantitation of BAEP parameters per treatment group. Interwave-interval between waves I-II in 16 days-old Gunn rat pups. Pups were randomized to receive saline (control), phenylhydrazine (phz) or sulfadimethoxine (sulfa), and were subsequently treated with saline or albumin (Alb). Values are mean ± SD. *p<0.05 compared to controls.

Plasma B_f concentrations

In Figure 4 plasma B_f concentrations are shown. In the hemolysis model, the Phz/Sal-treated animals had significantly higher B_f concentrations compared to controls (+27%; p<0.05). The Phz/Alb-treated animals had higher B_f concentrations compared to Phz/Sal-treated animals (+19%). In the displacement-model we found the same pattern for the B_f concentrations as for the UCB concentrations. The Sulfa/Sal-treated animals had significantly lower plasma B_f concentrations compared to controls (-81%; p<0.05). The Sulfa/Alb-treated animals had higher B_f concentrations compared to Sulfa/Sal-treated animals (+57%).

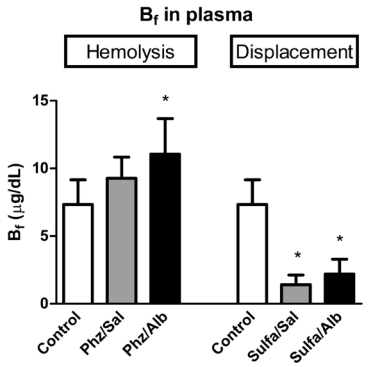

Figure 4. Plasma B_f concentrations.
Effects of no treatment (control) or albumin (Alb) on plasma B_f concentrations in as well the hemolysis (phz) as the displacement (sulfa) model for hyperbilirubinemia in 16-days old Gunn rat pups. Pups were randomized to receive saline (control), phenylhydrazine (phz) or sulfadimethoxine (sulfa), and were subsequently treated with saline or albumin (Alb). Values are mean ± SD. *p<0.05 compared to controls.

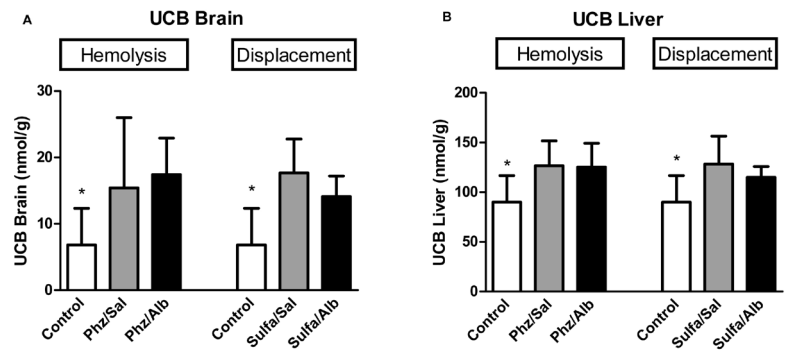

Figure 5. Tissue bilirubin levels.
Effects of no treatment (controls), or albumin (Alb) on brain (**A**) and liver (**B**) bilirubin levels in the hemolysis (phz) and displacement (sulfa) model for hyperbilirubinemia in 16-days old Gunn rat pups. For experimental setup, we kindly refer to the Methods section. Values are mean ± SD. *p<0.05 compared to treatment-groups.

Tissue UCB levels

In the hemolysis model, the control group had significantly lower UCB brain levels compared to animals treated with Phz/Sal and Phz/Alb (p<0.05; Figure 5A). In the displacement model, UCB brain levels are significantly lower in the control group compared to animals treated with sulfa, irrespective of its additional treatment with saline or albumin (p<0.05; Figure 5A). In both models, albumin treatment did not decrease UCB brain levels.
The liver UCB levels showed the same pattern as the brain UCB levels in both experimental models (Figure 5B).
Figure 6 shows that brain bilirubin levels correlated with IWI I-II in Gunn rat pups (y=14.29x -5.003; Spearman R=0.41; p<0.008).

Brain B_f concentrations

Figure 7 shows that in the hemolysis model, the Phz/Sal-treated animals had significantly higher brain B_f concentrations compared to controls (+193%; p<0.01). The Phz/Alb-treated animals had lower brain B_f concentrations compared to Phz/Sal-treated animals (-36%; NS). In the displacement model we found that the Sulfa/Sal-treated animals had significantly higher brain B_f concentrations compared to controls (+250%; p<0.001). The Sulfa/Alb-treated animals had lower brain B_f concentrations compared to Sulfa/Sal-treated animals (-20%; NS).

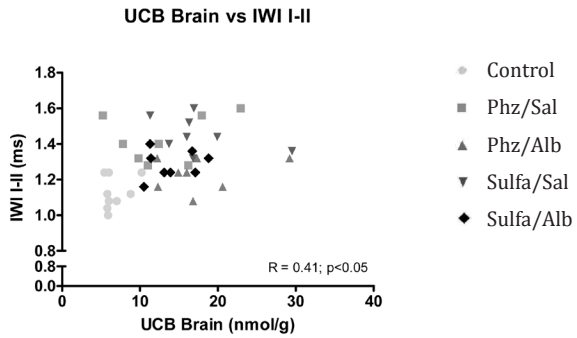

Figure 6. The correlation between brain bilirubin levels and interwave-interval I-II.
The correlation between brain bilirubin levels and interwave-interval I-II in 16-days old Gunn rat pups. Pups were randomized to receive saline (control), phenylhydrazine (phz) or sulfadimethoxine (sulfa), and were subsequently treated with saline (control) or albumin (Alb).

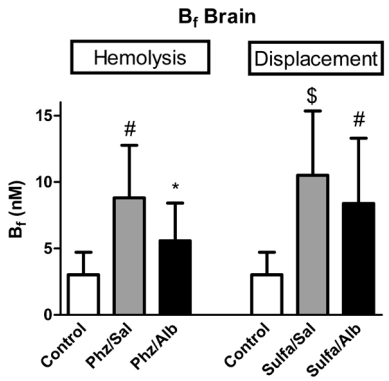

Figure 7. Brain free bilirubin concentrations.
Effects of no treatment (control) or albumin (Alb) on brain B_f concentrations in as well the hemolysis (phz) as the displacement (sulfa) model for hyperbilirubinemia in 16 days-old Gunn rat pups. Pups were randomized to receive saline (control), phenylhydrazine (phz) or sulfadimethoxine (sulfa), and were subsequently treated with saline or albumin (Alb). Values are mean ± SD. *p<0.05 compared to controls. #p<0.01 compared to controls. $p<0.001 compared to controls.

DISCUSSION

In this study we demonstrate in a functional assay that HSA treatment exerts neuroprotective activity in two models for unconjugated hyperbilirubinemia. The neuroprotective effect of HSA can be discerned by preventing the increase in interwave-interval I-II in BAEPs. The neuroprotective effect was apparent in both a hemolytic and a bilirubin displacement model. Our data underline the value of functional diagnostic testing, because biochemical analyses (B_f and UCB concentrations in plasma and brain) were not conclusive.

In plasma, most of the unconjugated bilirubin is bound to albumin and only a small fraction (<1%) is free. The rationale to use albumin as a treatment option for hyperbilirubinemia, is based on the hypothesis that *i.v.* albumin binds UCB within the plasma, presumably lowering B_f and preventing the translocation of bilirubin into the brain. The present study provided the "proof of concept". We used an albumin dose of 2.5 g/kg body weight, which is higher than what is infused in human neonates (*i.e.* 1 g/kg body weight). One animal study is known, in which the treatment of HSA is evaluated with BAEPs after induction of acute neurotoxicity by sulfa in Gunn rat pups.[2] In comparison to our study, in which we demonstrate the prevention of neurotoxicity, Shapiro showed that therapeutic intervention with HSA as late as 8h after acute bilirubin encephalopathy in this animal model promotes the recovery of neurophysiologic function as effectively as intervention at 2h. This indicates that a hypothesized "critical period" for recovery of auditory brainstem function after acute bilirubin encephalopathy may extend beyond 2h.[2] The protective role of HSA treatment has also been investigated in neonates. Two retrospective studies have shown reduced B_f levels in neonates with hyperbilirubinemia after HSA treatment.[3,4] Some protective effect of HSA treatment on the development of brain damage was suggested in a small cohort study, as measured by auditory brainstem responses.[5] However, other studies have failed to show the beneficial effects of HSA treatment.[6] The efficacy of HSA treatment in acute hyperbilirubinemia has never been established in a randomized control trial or under rigidly controlled conditions in a model system. Chan and Schiff speculated that in most instances the administration of albumin does not significantly improve the reserve albumin-binding capacity. It would seem that the use of albumin would only have a significant effect in those situations where the binding capacity of the infant is already compromised.[6] Only recently, Ahlfors *et al.* showed that auditory brainstem responses correlated better with B_f than total serum bilirubin concentrations in neonates.[25]

In Gunn rats, bilirubin neurotoxicity generally does not affect wave I from the BAEP recordings. Waves II and III become abnormal, displaying increased latency and decreased amplitudes.[12,26] Waves II and III are the most sensitive to bilirubin. In humans, waves I and II are generated by the auditory nerve, whereas in rats the auditory nerve only produces wave I.[27,28] The following wave, III in humans and II in rats is generated by the cochlear nucleus.[27,29] Multiple structures contribute to the generation of wave III in rat, although most probably it is primarily originating from contralateral structures in the superior olivary complex including the lateral leminiscus.[7] In our rat model BAEP waves I, II and III correspond to wave I-II complex, wave III and wave IV-V complex in

humans. Wave I in rats originates from the auditory nerve and wave II is from the cochlear nucleus.[7,29] In this study, the I-II interwave-interval was increased during acute bilirubin neurotoxicity in both the hemolysis (phz) model and the displacement (sulfa) model. Based on these data, it is likely that the cochlear nucleus is predominantly affected by bilirubin toxicity. This interpretation is supported by earlier data from Haustein *et al.*, who demonstrated that hyperbilirubinemia caused degeneration of excitatory synaptic terminals in the auditory brainstem of 14-20 days old Gunn rat pups.[30] Thereby, albumin can prevent this functional brain damage, shown in the absence of increased interwave-interval I-II.

We first evaluated the total plasma UCB concentrations in both models. In the hemolysis-model, the Phz/Sal-treated animals have significantly higher plasma UCB concentrations compared to controls. This is expected, since we increased the UCB-production by inducing hemolysis. Interestingly, the Phz/Alb-treated animals have significant higher plasma UCB concentrations compared to Phz/Sal-treated animals, compatible with redistribution of UCB to the intravascular space and the larger capacity of blood to bind neurotoxins, including UCB, after albumin treatment. Our data also underline that the *i.p.* administered albumin readily enters the bloodstream compartment and does not remain in the intraperitoneal cavity. Unfortunately, due to the experimental set-up in pups, we were not able to measure plasma albumin concentrations. Albumin is believed to exert its beneficial effects in the blood circulation. In a previous study in adult Gunn rats, we showed that HSA readily enters the plasma compartment after *i.p.* injections.[31] In accordance with the concept, the displacement model shows lower UCB concentrations compared to the hemolysis model. Contrary to the increased production of UCB in the hemolysis model, the lowering in plasma UCB concentrations in the displacement model is due to a translocation of UCB into the tissue. The Sulfa/Sal-treated animals have significantly lower plasma UCB concentrations compared to controls, since the rat's own albumin has a decreased capacity to bind bilirubin due to the binding of sulfa. As in the hemolysis-model, the Sulfa/Alb-treated animals also show higher plasma UCB concentrations compared to Sulfa/Sal-treated animals. We consider it likely that the same explanation holds as for the Phz/Alb-treated animals. Namely, the redistribution of UCB to the intravascular space and the larger capacity of blood to bind UCB after albumin treatment.

We evaluated the plasma B_f concentrations in the hemolysis model as well as the displacement model. Surprisingly, for B_f we saw the same pattern as for total plasma UCB. After albumin treatment, the plasma B_f increases in both models of acute hyperbilirubinemia. This result is in contrast to our hypothesis that albumin would reduce plasma B_f, thereby preventing its translocation into the brain. Several explanations are possible. Firstly, the peroxidase-method may not be a reliable method to measure plasma B_f, which would imply that our biochemical results may not be accurate. An alternative method to measure B_f might be the use of a fluorescently labeled fatty acid binding protein mutant (B_f probe), that allows direct monitoring of the equilibrium B_f concentration.[32] At the moment we conducted our study, the B_f probe was not yet commercially available. Secondly, there may be other mechanisms involved, which induce brain damage, besides the generally accepted theory of the translocation of free bilirubin into the brain. Thirdly, the high

supraphysiological albumin concentration may interfere with the plasma B_f analysis. Together, however, it does show that biochemical data on bilirubin seem to be inferior to functional analysis of toxicity in the (possibly) affected organ, in this case the auditory system. This is possibly due to the inherent difficulties to obtain precise measurements in specific body compartments.

To the best of our knowledge, this is the first study, in which free bilirubin concentrations and brain bilirubin levels are evaluated and compared to a functional test. The bilirubin-albumin displacement model is in accordance with our hypothesis, in that the Sulfa/Alb-treated animals have lower UCB brain levels than the Sulfa/Sal-treated animals, although this difference is not statistically significant. The lower UCB brain levels in the albumin-treated animals also correlate with their BAEPs, which are less abnormal. In the hemolysis-model, the Phz/Alb-treated animals tended to have higher brain UCB levels compared to Phz/Sal-treated animals, although the differences were not statistically significant. We speculate that this may be related to the development of the rat's BBB. In Wistar rats, higher levels of endogenous albumin can be found in all regions of the developing brain of rat pups aged 2, 7, 11 and 21 days, compared to the values in adults (45 and 90 days).[33] Postnatal synthesis of albumin in the rat brain was not identified as a possible source; instead, increased BBB was implicated as a culprit.[33] Two other studies showed that the BBB is not impermeable for albumin in early (rat) life: after administration of labeled albumin a higher concentration of labeled albumin is found in the immature rat brain compared to the adult rat brain.[34,35] In accordance with the concept, we noticed a correlation between UCB Brain and IWI I-II. Interestingly, Figure 6 also indicates that part of the variation in IWI I-II does not correlate with UCB Brain. A potential explanation for the results in the hemolysis model can be the higher permeability of the BBB. In our study, the Gunn rats were 16-days of age, and several other studies showed higher brain albumin levels at that age.[33-36]

The brain bilirubin levels in the displacement model are in accordance with our hypothesis. We demonstrate that when B_f plasma concentrations are lowered and BAEP recordings are normal, the brain UCB levels are also low (comparable to control levels). Apparently, more bilirubin will enter the brain upon induction of hemolysis, than by inducing displacement of bilirubin from albumin.

Finally, we show that albumin treatment non-significantly reduces B_f concentrations in brain, compared to saline treatment. The observed pattern mimics the B_f concentrations in plasma. This observation might, in part, explain the beneficial mechanism by which albumin protects from bilirubin-neurotoxicity.

In conclusion, albumin treatment is neuroprotective in acute hyperbilirubinemia in Gunn rat pups, irrespective of its induction by hemolysis or by bilirubin displacement from albumin. The discrepancy between BAEPs (functional results) and UCB brain levels (biochemical results), shows the importance of functional diagnostic tests, particularly in the field of unconjugated (free) bilirubin. Also, we show a possibly new phenomenon not based on bilirubin in brain or B_f, but based on the higher permeability of the BBB in rat pups. It seems worthwhile to further investigate this phenomenon and its potential influences on bilirubin induced neurological dysfunction in future studies. Present beneficial, functional results favor the clinical potency of albumin treatment to prevent or mitigate neurotoxicity due to severe neonatal hyperbilirubinemia.

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Chapter 5

POLYETHYLENE GLYCOL AND URSODEOXYCHOLIC ACID PREVENT SEVERE NEONATAL JAUNDICE IN GUNN RAT PUPS

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Submitted

ABSTRACT

Background: Neonatal jaundice carries the risk of neurotoxicity. Once jaundice is present, several therapies are available. Unfortunately, preventive strategies are limited. In adult rats jaundice can be treated by enhancing the fecal bilirubin excretion with polyethylene glycol (PEG) or ursodeoxycholic acid (UDCA).

Aim: To determine whether PEG or UDCA can prevent jaundice in a rat pup model.

Methods: We used Gunn rat pups, which have a deficiency of the bilirubin-conjugating enzyme UGT1A1, as a model for neonatal jaundice. We measured transcutaneous bilirubin (TcB) concentrations between postnatal days 1-21. At day 7 rats received either no treatment (control; n=28, or saline gavage; n=7), PEG (n=8) or UDCA (n=7) *via* gavage. TcB, plasma unconjugated bilirubin (UCB), and brain UCB levels were determined.

Results: The neonatal hyperbilirubinemic peak occurred in Gunn rat pups between days 15-18. PEG and UDCA significantly decreased this peak on day 15 by 28% and 41% ($p<0.05$) respectively, compared with controls. On day 18 both treatment groups were still significantly lower compared with controls (PEG -26%, UDCA -20%; both $p<0.05$).

Conclusion: We provide a proof of concept in a rat pup model for severe neonatal jaundice that PEG and UDCA can prevent jaundice.

INTRODUCTION

Unconjugated hyperbilirubinemia occurs in ~70% of term, and in almost all preterm infants. In the postnatal period the human newborn has an immature hepatic bilirubin conjugation and elimination system, in relation to increased bilirubin production after the transition from intrauterine-placental to neonatal-pulmonary oxygenation.[1] Accumulation of bilirubin in the body can have different causes but, irrespective of the cause, results from an imbalance between bilirubin production and excretion. Ultimately this results in the accumulation of excessive bilirubin in the circulation (hyperbilirubinemia) and subsequently in the skin, sclerae (jaundice), and other organs. The most dangerous location where bilirubin can accumulate is the central nervous system. Within specific locations in the central nervous system, unconjugated bilirubin (UCB) induces apoptosis and necrosis, which can lead to kernicterus and even death.[2-4]

Once jaundice is present, several therapies are available, such as phototherapy and exchange transfusion. Unfortunately, however, preventive strategies against neonatal jaundice are presently limited. Two preventive options are known, namely heme oxygenase inhibitors and intravenous immunoglobulins, but these preventive strategies are invasive and have considerable side effects.[5,6] Fecal excretion of bilirubin, or of its degradation products, constitutes the main excretory pathway of bilirubin for the body. We previously demonstrated that unconjugated hyperbilirubinemia could be treated in adult Gunn rats, the well-established animal model for unconjugated hyperbilirubinemia, by enhancing the fecal UCB excretion via two different strategies.

The first of these strategies involves stimulation of transepithelial UCB transfer from the blood into the intestinal lumen.[7-11] In hyperbilirubinemic Gunn rats, the majority of UCB enters the intestinal lumen *via* this pathway, rather than *via* the bile.[11] Transmucosal diffusion, and the subsequent fecal excretion of UCB can be achieved by accelerating the gastrointestinal transit by the laxative polyethylene glycol (PEG). PEG decreases plasma UCB concentrations and a strong correlation was demonstrated between reducing the gastrointestinal transit time and lowering the plasma UCB concentrations.[12] The second strategy to increase the fecal excretion of bilirubin was obtained by bile salt treatment. Bile salts can stimulate biliary excretion of unconjugated bilirubin in rats.[13] We previously showed that dietary administration of either ursodeoxycholic acid (UDCA) or cholic acid induced a rapid and sustained decrease in plasma UCB concentrations in adult Gunn rats. The mechanism involves stimulation of both biliary and transepithelial UCB translocation into the intestinal lumen, and its subsequent fecal disposal.[12]

The preventive capacity of these two strategies, PEG and UDCA, for neonatal jaundice is unknown. Prevention of hyperbilirubinemia would be especially useful in resource limited countries, where phototherapy and exchange transfusion are not always available.[14] In the present study we tested in Gunn rat pups, an animal model for severe neonatal jaundice, whether strategies known to enhance fecal excretion can prevent severe hyperbilirubinemia during neonatal jaundice. We provide a proof of concept in a rat pup model for severe neonatal jaundice that PEG and UDCA can prevent severe unconjugated hyperbilirubinemia.

ANIMALS, MATERIALS, AND METHODS

Animals

Homozygous Gunn rat pups (RHA/jj; 0-21 days old) from our breeding colony were used. The Gunn rats were housed per litter (n=6) and kept in an environmentally controlled facility. The pups were randomized per litter (n=6-10) to receive different treatment options. The dams were fed chow *ad libitum* and had free access to water. The Animal Ethics Committee of the University of Groningen (Groningen, The Netherlands) approved all experimental protocols.

Materials

Chemicals

Horseradish peroxidase type 1, D-glucose, glucose oxidase, and hydrogen peroxide were purchased from Sigma Chemical Co. (St. Louis, MO). PEG 4000 (Colofort ®) was obtained from Ipsen Farmaceutica BV (Hoofddorp, The Netherlands). The PEG solution we used (gavage solution) was obtained by dissolving one sachet (74 g) of PEG 4000 in 900 ml water. UDCA suspension 50mg/ml was obtained from the University Medical Center Groningen Pharmacy. The UDCA suspension was diluted with water to a concentration of 25 mg/ml.

Methods

Study design

In Gunn rat pups transcutaneous bilirubin concentrations (TcB) were measured daily from postnatal day 1 till day 21. At day 7 of age Gunn rat pups were randomized per litter to receive either no treatment (controls; n=28, saline; n=7), PEG (10 ml/kg of gavage solution; n=8) or UDCA (10 ml/kg of gavage solution; n=7) *via* gavage. Gavage was given twice a day *via* flexible feeding tubes. Blood was collected at day 7, 15, 18 and 21 by sacrificing 7 rats per time-point out of the control group (no treatment), for plasma UCB concentration determination. Plasma UCB concentrations were determined in these pups, to assess their correlation to TcB measurements. At day 21 of age, all animals were exsanguinated *via* a heart puncture and flushed *via* the same port with 10-20 ml NaCl 0.9% under isoflurane anesthesia. Liver and brain were subsequently collected for the determination of tissue bilirubin levels. These organs were snap frozen in liquid nitrogen, and immediately stored (wrapped in aluminum foil) at -80 °C until analysis.

Transcutaneous bilirubin measurements

The transcutaneous measurements were performed with the Minolta Airshield Jaundice Meter 103 (JM-103, Dräger Medical, Lübeck, Germany). The JM-103 determines the difference in skin reflectance between optical densities for light in the blue (450 nm) and green (550 nm) wavelength regions. By using two optical paths, the reflectance of melanin, dermal maturity, and hemoglobin from the superficial tissue can be deducted.[15] Regular calibration of the JM-103 following the

instructions of the manufacturer was ensured. TcB measurements were made at the head and the hip of the Gunn rat pups. Three consecutive measurements were taken and the mean was used (expressed in µmol/L).

Analytical Methods

Plasma analysis

Blood samples were protected from light, stored at -20 °C under argon directly after collection, and processed within 2 weeks. UCB and B_f were determined using a Zone Fluidics system (Global Flopro, Global Fia Inc, WA), as previously described by Ahlfors *et al.*.[16]

Tissue bilirubin analysis

Tissue bilirubin content was determined using HPLC with diode array detector (Agilent, Santa Clara, CA) as described earlier.[17] Briefly, 300 pmol of mesobilirubin in DMSO (used as an internal standard) was added, and samples were homogenized with glass dust using glass rod. Bile pigments were then extracted into chloroform/methanol/hexane (10:5:1) solution at pH 6.0, and subsequently extracted in a minimum volume of methanol/carbonate buffer (pH 10.0) to remove contaminants. The resulting polar droplet (extract) was loaded onto C-8 reverse phase column (Phenomenex, Torrance, CA) and separated pigments were detected at 440 nm. The concentration of bilirubin was calculated as nmol/g of wet tissue weight. All steps were performed under dim light in aluminum-wrapped tubes. We did not specifically measure bilirubin deposition in the brain nuclei, because of the still small sizes of rat pup brains, but rather measured bilirubin concentration in total brain.

Statistical analysis

Normally distributed data that displayed homogeneity of variance (by calculation of Levene's statistic) were expressed as mean ± SD, and analyzed with parametric statistical tests. We calculated the area under the TcB curves per rat from day 7 till day 21 of age. Repeated measurement analysis of variance (MANOVA) with post-hoc Bonferroni correction was performed for comparisons between groups. The level of significance was set at $p<0.05$. The mean TcB and the mean difference between the TcB and UCB measurements was calculated and illustrated in a Bland Altman plot for all measurements.[18]Analyses were performed using SPSS Statistics 20.0 for Windows (SPSS Inc., Chicago, IL).

RESULTS

Validation of the model

The transcutaneous bilirubin analysis with the Minolta Airshield Jaundice Meter 103 has not been validated in Gunn rat pups. We first evaluated if we reliably and reproducibly could measure bilirubin transcutaneously in Gunn rat pups. We determined the correlation and agreement between TcB and plasma UCB concentrations.

Figure 1 shows the transcutaneous bilirubin concentrations in untreated Gunn rat pups during the first three weeks of life. The TcB concentrations measured on the head of the pups were virtually identical as those measured on the hipbone. Peak TcB concentrations were reached on day 18 of age (340 µmol/L) with a plateau from day 15 till 18 of age (328-340 µmol/L).

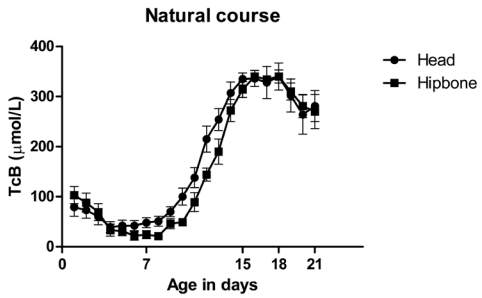

Figure 1. Natural course of transcutaneous bilirubin concentrations.
Course of transcutaneous bilirubin concentrations in Gunn rat pups. Rats were daily measured from day 1 till day 21 of age. TcB measurements were made at the head and the hipbone of the Gunn rat pups. Three consecutive measurements in µmol/L were taken and the mean was used. The transcutaneous measurements were performed with the Minolta Airshield Jaundice Meter 103. Values are mean ± SD.

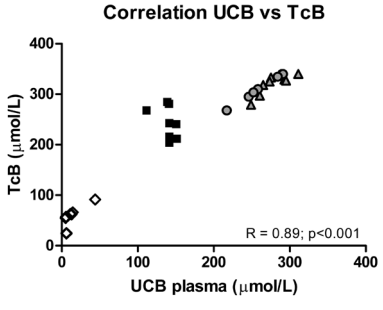

Figure 2. The correlation between plasma bilirubin and transcutaneous bilirubin concentrations.
The correlation between plasma bilirubin (UCB) and transcutaneous bilirubin (TcB) concentrations in Gunn rat pups. TcB was measured daily from day 1 till day 21 of age. At different time points (day 7, day 15, day 18, and day 21) Gunn rat pups (n=7 per group) were sacrificed to measure plasma UCB concentrations. ◇ Day 7, △ Day 15, ○ Day 18, ■ Day 21.

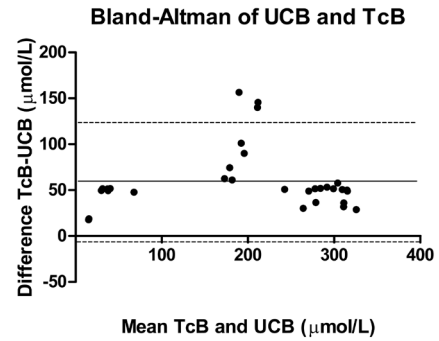

Figure 3. The agreement between plasma bilirubin and transcutaneous bilirubin concentrations depicted in a Bland-Altman plot.
The X-axis shows the mean of Z-scores of TcB and UCB, and the Y-axis shows the difference between Z-scores of TcB and UCB. The horizontal line represents the mean difference (59 µmol/L), the dotted lines represent the 95% confidence interval (-6 to 125 µmol/L).

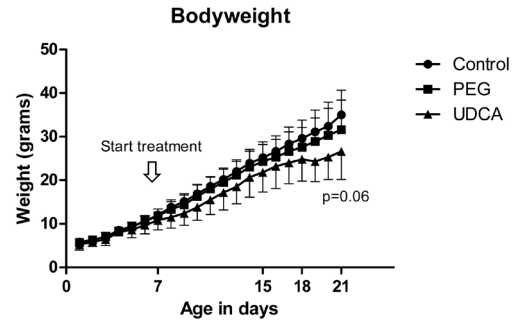

Figure 4. Course of bodyweight between different treatment groups.
Course of bodyweight of Gunn rat pups from day 1 till day 21 of age. Gunn rat pups were randomized to receive either no treatment (controls; n=7), PEG (10 ml/kg of gavage solution; n=8) or UDCA (10 ml/kg of gavage solution; n=7) *via* gavage. Gavage was given twice a day *via* flexible feeding tubes. Values are mean ± SD.

Figure 2 shows the correlation between plasma UCB concentrations and TcB measurements. At day 7, day 15, day 18, and day 21 plasma UCB concentrations were determined. Plasma UCB concentrations correlated strongly with TcB measurements (R=0.89; p=0.01). Figure 3 shows the agreement between TcB and UCB measurements. TcB values were in general 50 µM higher than the UCB concentrations. Figure 2 and 3 indicate that particularly at day 21 the two measurements seem to diverge, when compared with the other time points.

Effects of PEG or UDCA treatment on bodyweight

Figure 4 shows the effects of the different treatments on the bodyweight of the pups between postnatal day 1 and 21. The bodyweight of the UDCA group was the lowest throughout the whole experiment, although it did not reach statistical significance (p=0.06) compared with the control group. From day 18 onwards this lower bodyweight became more prominent. During our experiments the Gunn rat pups in the UDCA group had visible diarrhea and seemed less active than littermates, receiving either saline or PEG (data not shown).

PEG or UDCA treatment mitigates the hyperbilirubinemic peak

Figure 5 shows the course of transcutaneous bilirubin between different treatment groups in Gunn rat pups. From day 12 onward significant differences between the treatment groups became apparent. On day 12 and 13 the TcB measurements in the UDCA group were significantly lower compared with either the control or the PEG group. From day 14 till day 18 both the PEG and the UDCA group had significantly lower TcB concentrations than the control group. The hyperbilirubinemic peak in controls between day 15 and day 18 of age, was prevented by either PEG or UDCA. At day 15, PEG and UDCA decreased TcB by 27%, and even by 41%, respectively, compared with controls (each $p<0.05$). At day 18 both groups were still significantly lower compared with controls (PEG -26%; $p<0.05$, UDCA -20%; $p<0.05$). On day 19 only the UDCA group shows significant lower TcB measurements compared with the control group. At day 21 the TcB concentrations in the control group decreased (-30% vs day 15), reaching similar values as the two experimental groups in which the TcB stabilized after day 15.

The area under the curve (AUC) from day 7 till day 21 of age was significantly lower in both the PEG (AUC 2424±154 day*µmol/L; -20%) and the UDCA group (AUC 2125±143 day*µmol/L; -30%), compared with controls (AUC 3015±105 day*µmol/L; each $p<0.01$). The AUC in the UDCA group was also significantly lower than that of the PEG group ($p<0.01$; Figure 5).

Tissue UCB levels

Figure 6 shows the brain and liver bilirubin levels per treatment group at day 21. The PEG (4.7±0.7 nmol/g) and the UDCA group (5.4±1.0 nmol/g) showed no significant difference in brain UCB levels compared with controls (4.5±1.2 nmol/g). Similarly, the liver bilirubin levels were not significantly different between the three groups.

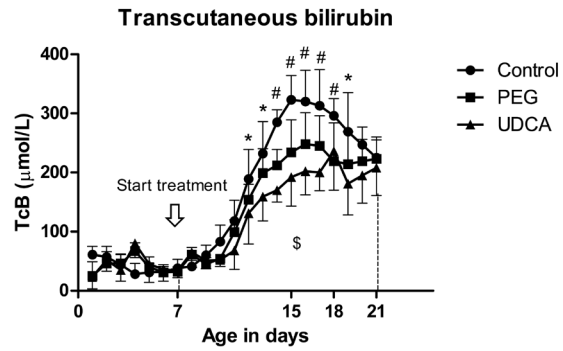

Figure 5. Course of transcutaneous bilirubin between different treatment groups.
Course of transcutaneous bilirubin (TcB) of Gunn rat pups from day 1 till day 21 of age. TcB was measured daily from day 1 till day 21 of age. At day 7 of age Gunn rat pups were randomized to receive either no treatment (controls; n=7), PEG (10 ml/kg of gavage solution; n=8) or UDCA (10 ml/kg of gavage solution; n=7) *via* gavage. Gavage was given twice a day *via* flexible feeding tubes. Values are mean ± SD. *p<0.05 UDCA compared with controls. #p<0.05 PEG/UDCA compared with controls. $p<0.01 AUC day 7-21 PEG/UDCA compared with controls.

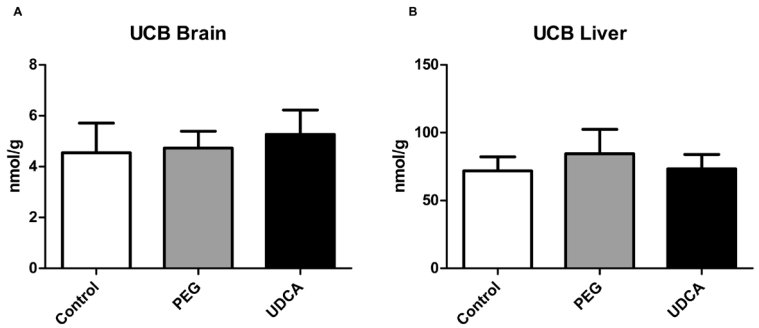

Figure 6. Tissue bilirubin levels at 21 days of age.
Brain (**A**) and liver (**B**) bilirubin levels in 21-days old Gunn rat pups. Gunn rat pups were randomized to receive either no treatment (controls; n=7), PEG (10 ml/kg of gavage solution; n=8) or UDCA (10 ml/kg of gavage solution; n=7) *via* gavage. Gavage was given twice a day *via* flexible feeding tubes. Values are mean ± SD.

DISCUSSION

In this study we provide a proof of concept in a rat model for severe neonatal jaundice, that treatment with either PEG or UDCA can prevent severe unconjugated hyperbilirubinemia. The prevention of bilirubin accumulation in plasma is probably due to the induction of the fecal disposal of bilirubin.

Vreman *et al*. were the first to demonstrate that TcB measurements could be successfully performed on newborn rat pups.[19] These scientists showed that measurements made at the appropriate site, namely on the head and lower back (after shaving off the fur) closely correlated with total serum bilirubin (TSB) concentrations during the first week of life.[19] In our study, we confirmed this observation using a different type of jaundice meter, namely the Minolta Airshield Jaundice Meter 103. Similar to Vreman *et al.* we found that TcB concentrations peaked between postnatal day 15 and day 18 in Gunn rat pups. Also, we observed a similar decrease in TcB values during the first four postnatal days. The decrease in TcB values corresponded with similar observations in TSB concentrations between 30 to 96h of life.[20] The slight decrease has been attributed to the equilibration between bilirubin production, catabolism, derivatization by mixed function mono-oxygenase[21], and to biliary excretion of unconjugated bilirubin into the intestine[7], and possibly, the enterohepatic circulation.[19]

In our study TcB measurements strongly correlated with plasma UCB concentrations, which is in line with previous data.[19] The Bland-Altman plot showed an acceptable agreement between "the gold standard", *i.e.* plasma bilirubin concentrations, and TcB measurements, at least when used for screening purposes. Based on these data we concluded that the JM-103 was sufficiently reliable to use as a follow-up instrument during our experiments. However, our results indicated that particularly at day 21 the two measurements seem to diverge, when compared with the other time points. Theoretically, age dependent changes in the skin might contribute to this observation. Another major change at this time point involves a dynamic change in diet, from predominantly milk feeding towards chow. We speculate that this could also interfere with the bilirubin homeostasis.
At day 7 of age we started treatment with PEG or UDCA *via* oral gavage. Treatment with either PEG or UDCA decreased and delayed the hyperbilirubinemic peak in Gunn rat pups. Remarkably, during the first 5-6 days of treatment, no difference in the transcutaneous bilirubin measurements was observed between the PEG, UDCA, and control group. From postnatal day 12 onwards, however, the curves started to diverge. An explanation might be, that the two treatment strategies need some time to exert their preventive effect in rat pups. Theoretically, one could test this hypothesis by starting treatment earlier, *i.e.* before 7 days of age. However, starting (and gavaging) earlier may be harmful to the extremely small rat pups and probably not necessary. By starting treatment at 7 days of age, we now already show that the harmful neurotoxic bilirubin peak can be prevented.

In accordance with the literature, the physiological hyperbilirubinemic peak with levels above 300 μM occurred between day 15 and day 18 of age. The two treatment strategies, PEG and UDCA, completely prevented the severe hyperbilirubinemia: the bilirubin concentrations in these treatment groups did not exceed 250 μM. We have no reason to believe that PEG and UDCA only delayed the hyperbilirubinemic peak. First, we showed that both therapies keep the TcB concentrations lower during the peak-period from day 15 and 18, and afterwards stabilize the concentrations till day 21 of age. At day 21 the TcB and plasma bilirubin concentrations were similar in the three groups. Second, we found no differences in brain or liver bilirubin levels between the three treatment groups at day 21. This observation indicated that the brain levels closely corresponded with the plasma concentrations, and that the treatment groups did not interfere with this correspondence. In other words, the prevention of severe hyperbilirubinemia in the treatment groups can reasonably be extrapolated to lower accumulation of bilirubin in the brain between day 15 and 18. It seems reasonable to assume that the preventive effect of PEG and UDCA is due to the same mechanism shown in adult Gunn rats, namely enhancing the fecal excretion of UCB. In the present study, however, we were not able to quantitatively collect feces, and determine the fecal bilirubin disposal from the rat pups.

Two other strategies to mitigate the development of severe jaundice are available, heme oxygenase inhibitors, *i.e.* porphyrins, and immunoglobulins. Bilirubin is a product of heme degradation, and the rate limiting step in its formation is controlled by the enzyme heme-oxygenase. Presently, the only synthetic inhibitor that is approved by the Food and Drug Administration for clinical use is Tin-mesoporphyrin (SnMP). Over a 10-year period, Kappas *et al.* conducted a number of randomized controlled clinical trials with SnMP in infants with different forms of newborn jaundice.[5] Kappas *et al.* concluded that 6 μmol/kg of bodyweight is an appropriate single dose of SnMP.[22] Moreover, they monitored treated infants for periods ranging up to 5 years, and observed no significant early or late side effects of SnMP treatment. SnMP showed to be a preventive strategy in 80 newborns in whom bilirubin levels were already close to the level requiring phototherapy (~330 μM). One single dose of SnMP rapidly blocked the progression of jaundice, and none of the infants required supplemental phototherapy.[23] Nevertheless, administration of SnMP has not become a routine clinical strategy. As with the administration of any drug, there can be adverse metabolic consequences of SnMP therapy. In combination with phototherapy SnMp is phototoxic, and SnMP interferes with other important metabolic pathways.[24] Taking into account the side effects, at present the use of SnMP has been reserved for neonates who are at especially high risk for developing bilirubin-induced neurologic dysfunction, such as those requiring exchange transfusion.[24]
The other preventive treatment strategy, intravenous immunoglobulin (IVIG) administration, has sporadically been used for the treatment of hemolytic diseases of the newborn to avoid exchange transfusion since the early 1990s.[25] The exact mechanism of action is unknown but it is thought to inhibit hemolysis by blocking antibody receptors on red blood cells.[26] The 2004 American

Academy of Pediatrics guidelines recommend that in isoimmune haemolytic disease IVIG (0.5–1 g/kg over 2h) should be administered if the TSB increases despite intensive phototherapy, or if the TSB level is within 34–51 μmol/L (2–3 mg/dL) of the exchange level.[27] The use of IVIG to avoid exchange transfusion in hemolytic diseases of the newborn has been explored in seven randomized controlled trials and has been meta-analyzed in a Cochrane review.[28] All randomized controlled trials showed that IVIG significantly reduced the need for exchange transfusion.[28-31] Serious side effects were very rare but include hypersensitivity and anaphylaxis. For both, porphyrins and IVIG, a disadvantage is the fact that both treatment options are invasive in contrast to the presently tested potential strategies, PEG or UDCA treatment.

In clinical practice PEG has been used to prepare bowels for endoscopy.[32] In the 1990s PEG became available as osmotic laxative for the treatment of chronic constipation.[33] Currently it is one of the most widely prescribed laxative agents. Long-term treatment with PEG is believed to be safe and highly effective in adults[34] as well as in children.[35,36] UDCA is a treatment option for adult patients with chronic cholestatic liver disease.[37] UDCA treatment is also used in pediatric patients with cystic fibrosis liver disease[38], and in children with progressive familial intrahepatic cholestasis.[39] Finally, UDCA can be used in premature infants to prevent and treat cholestasis due to total parental nutrition.[40] A relatively common side effect of PEG or UDCA treatment is diarrhea.
In our study, the UDCA treatment seemed more aggressive than the PEG treatment. Diarrhea was observed in Gunn rat pups treated with UDCA, but not in PEG treated rats. In addition, the UDCA group tended to have a lower bodyweight, compared with controls or PEG treated animals. We did not evaluate whether a lower dose of UDCA exerts a similarly preventive effect, but with fewer or less severe side effects.
To address to what extent present results in rats can relate to the human neonates, the treatment dosages of PEG or UDCA in infants should be compared with the preventive dosages we administered to the Gunn rat pups. For infants between 6 and 12 months with constipation, the advised PEG dosage is 4000 mg/kg/day. Compared with our preventive treatment in rats, the dosage given to infants is several fold higher. The Gunn rat pups received 1400 mg PEG/kg bodyweight/day. Our preventive dosage of UDCA in rats is higher than the treatment dosage used in infants. For infants the dosage for UDCA treatment is, dependent on the underlying pathology, ~15 mg/kg/day. For our preventive treatment in rat pups we used a dosage of 500 mg UDCA/kg bodyweight/day. Therefore, we like to underline that the present results should be considered as a "proof-of-principle". We did not evaluate an optimal dose for rat pups, but used a similar dosage of PEG and of UDCA that had been proven effective in adult Gunn rats.[11,12]
If presently described effects are also found in clinical studies, PEG or UDCA treatment may especially be valuable in situations where phototherapy and exchange transfusion are not available, or difficult to arrange, for example in resource limited countries.[14] In the Western world, PEG and

UDCA are also possible cheap and safe preventive strategies for severe jaundice. We think that our results emphasize the need to assess the use of PEG and UDCA as preventive treatment option for unconjugated hyperbilirubinemia in randomized controlled clinical trials.

Taken together, our data show that oral administration of either PEG or UDCA can prevent severe hyperbilirubinemia in Gunn rat pups. Our study underlines the need to critically evaluate the use of PEG and UDCA as preventive treatment options in jaundiced newborns in clinical trials. These studies will, hopefully, allow to further decrease the incidence of bilirubin-induced brain damage world-wide in the future.

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Chapter 6

FREE BILIRUBIN AND THE BILIRUBIN/ALBUMIN RATIO IN PRETERM INFANTS

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Submitted

ABSTRACT

Objectives: Free bilirubin (B_f), the fraction of unconjugated bilirubin not bound to albumin, is associated more closely with bilirubin neurotoxicity than total serum bilirubin (TSB). However, B_f measurements are not routinely available. To estimate B_f, one might use TSB or, possibly more accurately, the bilirubin/albumin (B/A) ratio. We described the postnatal course of TSB, B/A ratio, B_f, and the association binding constant (K_a) in preterm infants. We analyzed the potential added value of the B/A ratio for estimating B_f.

Study design: We determined B_f and B/A ratio of preterm infants daily during the first ten postnatal days. B_f was measured using the peroxidase method and K_a was calculated. We evaluated the relationship between TSB, B_f, and B/A ratio by a multilevel model and by mass action equation. Data were expressed as mean (±SD) or [±SEM].

Results: We studied 72 infants (gestational age 29.2 weeks (±1.6), birth weight 1226 gram (±288)). Peak TSB concentrations were 170 μmol/L [±6], and peak B/A ratios were 5.4 μmol/g [±0.2], each occurring on the third day. Peak B_f concentrations of 49 nmol/L [±5] occurred on the fourth day. A large variation for K_a was found, not affected by risk factors or phototherapy. The B/A ratio did not prove to narrow the ranges of B_f more than TSB.

Conclusions: Bilirubin-albumin binding is highly variable postnatally. The B/A ratio does not outperform TSB alone for estimating B_f. We recommend to make B_f measurements routinely available to individualize the clinical approach of jaundiced preterm infants.

INTRODUCTION

Neonatal jaundice occurs in nearly all preterm newborns due to the transient postnatal imbalance in unconjugated bilirubin (UCB) production and excretion. As UCB accumulates, it distributes between the vascular and extravascular compartments (*i.e.* brain), and may cause bilirubin encephalopathy (BE). The risk of BE increases when concurrent conditions such as hemolysis, prematurity, or sepsis are present. The total serum or plasma bilirubin concentration (TSB) is the standard clinical measurement used to estimate the risk of BE. In line with this, management of infants with hyperbilirubinemia is based on TSB, although it has been demonstrated repeatedly in animal experiments and clinical studies that TSB is an unreliable parameter when it comes to predicting BE.[1-4]

TSB represents the total bilirubin load present in plasma, and UCB in plasma binds to plasma albumin (Alb), which prevents extravasation. In general, less than 1% of UCB is unbound or "free" bilirubin (B_f). B_f has the ability to move extravascular and to cross the blood-brain barrier. The plasma concentration of B_f may better reflect the risk of actual brain tissue exposure to bilirubin than TSB. B_f would therefore be the best vascular measure for assessing the risk of BE. This has dramatically been illustrated by the increased incidence of BE in newborns given drugs that impaired plasma bilirubin binding, resulting in abnormally high extravascular UCB levels and BE at "low" plasma TSB levels.[5]

Currently, measuring B_f is not routinely incorporated in clinical practice. This is mainly due to the fact that FDA-approved commercial instruments are not available. As an alternative approach, the plasma bilirubin/albumin (B/A) ratio has been suggested as surrogate parameter for B_f, since Alb measurements are clinically available, and plasma concentrations of B_f are determined by TSB, the Alb concentration, and their equilibrium association binding constant (K_a).[6] However, K_a is influenced by many (patho)physiological conditions and drugs. A range of B_f values would therefore be associated with any TSB treatment threshold (*e.g.* 340 μmol/L) for a given population of jaundiced newborns, with the highest B_f at the TSB threshold occurring at the lowest Alb and K_a for the population, and the lowest B_f at the TSB threshold occurring at the highest Alb and K_a. Measuring both TSB and Alb (*e.g.* the B/A ratio) might narrow the range of possible B_f values, and therefore be a better intervention guideline than TSB alone.[7]

The guideline issued by the American Academy of Pediatrics for the management of healthy, jaundiced newborns of ≥35 weeks' gestation with hyperbilirubinemia, recommends using the B/A ratio when an exchange transfusion is considered.[8] However, the degree to which both measures would narrow the range of B_f values depends on the variability of K_a in the population. Information on the postnatal course of the B/A ratio and B_f in preterm infants is essential to calculate K_a, but these data are not at hand.

In order to determine the added value of using the B/A ratio in estimation of B_f values compared to using only TSB, this study details B/A ratio, B_f concentrations, and the variability of K_a during the first ten postnatal days in preterm infants.

MATERIALS AND METHODS

This prospective study was part of the Bilirubin Albumin Ratio Trial (BARTrial, ISRCTN 74465643), and was approved by the Medical Ethics Committee of the University Medical Center Groningen. Written, and informed consent was given by the parents of all the infants that participated.

Patients

Newborns ≤32 weeks of gestation admitted to the Beatrix Children's Hospital, University Medical Center Groningen within 24 hours after birth were studied. Exclusion criteria were major congenital malformations, clinical syndromes or chromosomal abnormalities likely to affect neurodevelopmental outcome, and death within the first ten days after birth. Treatment was administered in accordance with Dutch guidelines, and depended on birth weight and the presence of specified risk factors.[9]

An infant was considered at high risk of bilirubin neurotoxicity if one or more of the following criteria were present: birth weight < 1000 g, asphyxia (Apgar score at 5 minutes: <3), hypoxemia (PaO_2 <5.3 kPa for more than two hours during the last 24 hours), acidosis (pH <7.15 for more than one hour during the last 24 hours), hemolysis (positive Coombs' reaction), sepsis needing vasopressor drugs, meningitis, clinical or central nervous system deterioration, intracranial hemorrhage of grade II (Papile grading), or higher.[9]

Measurement of TSB, B_f, and Alb

Blood samples were collected daily in EDTA tubes to determine infants' TSB, B_f, and Alb concentrations. They were centrifuged and the plasma was separated, protected from light, and stored at -80 °C under argon. TSB and B_f were measured using a Zone Fluidics system (Global Flopro, Global Fia Inc, WA), which uses the peroxidase method and minimal sample dilution.[10]

B_f was measured at two peroxidase concentrations to correct for rate limiting dissociation of UCB from albumin as previously described.[8]

To minimize (and quantify) the variance in K_a resulting from the test itself, a control sample (*i.e.* a pooled blood sample of the studied patients) was measured every day before running the study-samples. In addition, K_p was measured every week, after preparation of a new enzyme stock solution. Tests were performed daily; 25-30 samples were measured per run.

Albumin was measured spectrophotometrically using a P800 unit of a modular analytics serum work area from Roche Diagnostics Ltd. (Basel, Switzerland).

Calculation of K_a

B_f (at clinically relevant B/A ratios where TSB – B_f = ~TSB) is a function of TSB, Alb, and K_a according to the following equivalent mass action equations:

$$B_f = \frac{TSB}{K_a(Alb - TSB)} \quad \text{(Equation 1, 2 unknown variables)}$$

$$B_f = \frac{\frac{TSB}{Alb}}{K_a(1 - \frac{TSB}{Alb})} \quad \text{(Equation 2, 1 unknown variable)}$$

K_a was calculated from each daily measurement of TSB, B_f, and Alb. The variability in K_a for each infant, and for the population was assessed by calculating and comparing the within and between days mean, SD, SEM, and range for K_a. The day to day change in K_a was assessed because of evidence suggesting K_a increases over the first week of life.[11]

Separate analyses were performed to assess K_a in specific predefined subgroups based on birth weight (BW) and gestational age (GA), or the presence of risk factors, as stated in the Dutch guidelines.

Calculation of the range of B_f associated with TSB and with the B/A ratio

The range of B_f values obtained using TSB alone on each day were calculated by substituting the highest TSB, lowest Alb and lowest K_a (highest possible B_f), and the lowest TSB, highest Alb, and highest K_a (lowest possible B_f) into the mass action equation. The range of B_f obtained using the B/A ratio on each day was calculated by substituting the highest B/A ratio with the lowest K_a (highest possible B_f), and the lowest B/A ratio with the highest K_a (lowest possible B_f) into the mass action equation.

Statistical analysis

Normally distributed data, that displayed homogeneity of variance (by calculation of Levene's statistic) were summarized as mean ± SD and analyzed with parametric statistical tests. These analyses were performed using PASW Statistics 18.0 for Windows software (SPSS Inc., Chicago, IL). Separate analyses were performed to assess K_a in specific predefined subgroups based on BW, GA, and risk factors as stated in the Dutch guidelines. Furthermore, the effect of phototherapy (PT) on K_a was analyzed.

Concentrations of TSB, B_f, and the B/A ratio were summarized as mean ± SEM and analyzed with multilevel statistics. To detect differences in B_f over time between the different GA and BW categories, taking the B/A ratio into account, we built a multilevel model in which measurements at different days (level 1) were nested within subjects (level 2), thereby taking into account any

dependence between measurements. This multilevel analysis allows more accurate statistical testing than the standard repeated-measures ANOVA approach, because it allows unequal numbers of observations per subject, and it does not assume equality of group variances.[12]

For our study population, such a model would consist of numerous predictors. Here, we chose to use GA, BW, and postnatal days as predictors. For BW group designation the cut-off was set at 1250 grams, based on the Dutch intervention thresholds for treatment, and the higher risk of hyperbilirubinemia in these infants in the Netherlands. For GA we chose a cut-off of 29 weeks, based on the data distribution in our study population. Based on the postnatal course of B_f, we reduced the ten predictors, each representing one postnatal day, to three predictors representing day one, days two to four, and days five to ten.

This resulted in a model of 24 predictors, *i.e.* three time periods times two GA categories times two BW categories times two B/A ratio categories (with and without B/A ratio). Subsequently, the intercept was defined as a subject on day 1, with a GA of >29 weeks, and a BW of >1250 gram, and not taking the B/A ratio into account. To determine whether the B/A ratio was a better predictor for B_f than TSB alone, and which of the B/A ratio components, *i.e.* TSB or Alb, was the better predictor, we used the same model as described above, and replaced the B/A ratio by either TSB or Alb.

To test for differences between an estimated mean and the intercept we used a *t* test[13], and to test for differences between two estimated means we used a chi-square test. For all multilevel statistical analyses we used MLwiN 2.25 (University of Bristol, Bristol, UK).

RESULTS

Demographic data

Seventy-two infants were included in this study. Their mean GA was ~29 weeks with a BW of ~1200 grams. The clinical characteristics, listed in Table 1, show a relative low percentage of risk factors.

Postnatal course of TSB, B_f, and B/A ratio values

Figure 1A depicts the postnatal course of TSB. The TSB concentration was highest on day three (170 µmol/L [±6]). We found a statistically significant increase in TSB concentrations up to day three ($p<0.001$) followed by a statistically significant decrease between days four and five, and days six and seven ($p<0.05$). Figure 1B shows the postnatal course of the B/A ratio. In line with the postnatal course of the TSB concentrations, the B/A ratio was highest on day three (5.4 µmol/g [±0.2]). For the B/A ratio, we found a statistically significant increase up to day three ($p<0.05$), and a significant decrease from days four to eight ($p<0.05$). Figure 1C shows the postnatal course of B_f. The B_f concentration was highest on day four (49 nmol/L [±5]). We found a statistically significant increase between days one and two ($p<0.05$), but no statistically significant change between days two, three, and four. A statistically significant decrease was found between days four and five ($p<0.05$). Table 2 shows the binding data statistics of all included patients per day.

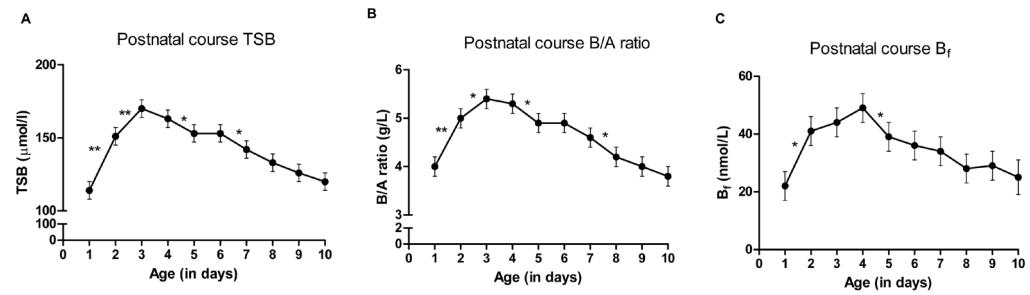

Figure 1. Postnatal course of TSB, B_f, and B/A ratio values.
A. Postnatal course of total serum bilirubin (TSB). TSB is expressed in µmol/L; 1 mg/dL = 17.1 µmol/L bilirubin.
B. Postnatal course of Bilirubin/Albumin (B/A) ratio. B/A ratios are expressed in µmol/g; multiply by ~ 0.066 to convert to B/A molar ratios.
C. Postnatal course of free bilirubin (B_f) concentrations in nmol/L.
Data are shown as mean ± SEM. P-values indicate significant differences between two following days. *p<0.05, **p<0.001.

Bilirubin-albumin binding affinity

Figure 2A depicts the postnatal course of K_a. Overall, the lowest K_a value was found on the fourth postnatal day (126 L/µmol [±12]). K_a gradually increased thereafter. To determine if the variability of K_a would be different in low and high risk newborns, we selected a sample size of ten preterm infants without and with risk factors. Figure 2B shows the postnatal course of K_a in ten low risk newborns (*i.e.* clinical stable newborns with a BW >1250 gram and without risk factors).

Table 1. Demographic and delivery characteristics.

Characteristics	Infants (N=72)
Gestational age in wks, mean (SD)	29.2 (1.6)
Birth weight in g, mean (SD)	1226 (288)
Male/Female	38/34
Prenatal steroids (%)	
Yes	38/68 (53%)
No	6/68 (9%)
Not completed	24/68 (35%)
Birth trauma, total (%)	6/72 (8%)
Caput succedaneum/cephalic hematoma	1/72 (1%)
Other hematoma	5/72 (7%)
Other bruising	0/72 (0%)
Apgar score < 3 at 5 min (%)	0/72 (0%)
Coombs (%)	
Positive reaction	0/72 (0%)
Negative reaction	42/72 (58%)
Unknown	30/72 (42%)
Irregular antibodies child (%)	
Positive	0/72 (0%)
Negative	61/72 (85%)
Unknown	11/72 (15%)
Irregular antibodies mother (%)	
Negative	68/68 (100%)
Sepsis (%) requiring volume expansion or vasopressants	3/72 (4%)
Hypoxemia (%)	2/72 (3%)
Acidosis (%)	0/72 (0%)
Meningitis, positive liquor culture (%)	1/72 (1%)
Intracerebral hemorrhage > grade II (%)	3/72 (4%)

Data are expressed as n/N (%), except for gestational age, birth weight, and the male/female ratio.

Table 2. Binding data statistics of all included patients per day.

Day	TSB Mean (µM) SEM (n) Range	Alb Mean (g/dL) SEM (n) Range	B/A ratio Mean (µM /g) SEM (n) Range	B/A ratio* Mean (M) SEM (n) Range	B_f Mean (nM) SEM (n) Range	K_a Mean (L/µM) SEM (n) Range
1	119.1	29.2	4.2	0.3	20.6	184.7
	4.4 (49)	0.6 (47)	0.2 (45)	0.09 (45)	2.3 (48)	17.2 (39)
	55-206	19-35	1.9-7.4	0.1-0.5	2-74	45-395
2	151.9	30.6	5.0	0.3	40.3	140.6
	4.6 (65)	0.6 (71)	0.2 (65)	0.1 (65)	4.5 (65)	12.9 (63)
	74-244	22-40	2.2-8.9	0.1-0.6	6-197	23-399
3	171.8	32.5	5.5	0.4	42.0	130.4
	5.6 (67)	1.0 (70)	0.2 (65)	0.09 (65)	4.0 (66)	11.4 (63)
	82-304	21-89	3.4-11	0.2-0.6	8-167	14-392
4	161.7	32.2	5.2	0.3	42.0	126.0
	5.7 (66)	0.6 (68)	0.2 (63)	0.1 (63)	3.4 (66)	11.5 (62)
	58-256	22-42	1.9-9.8	0.1-0.6	10-112	29-344
5	154.2	32.0	5.0	0.3	35.8	140.5
	5.1 (70)	0.6 (67)	0.2 (67)	0.1 (67)	3.6 (70)	12.5 (62)
	47-249	18-42	1.3-8.9	0.1-0.6	4-184	28-399
6	153.7	31.8	5.0	0.03	33.9	131. 6
	5.4 (67)	0.7 (70)	0.2 (66)	0.1 (66)	3.4 (67)	10.5 (59)
	38-242	17-44	1.0-8.9	0.1-0.6	4-140	40-379
7	144.5	31.5	4.7	0.3	31.7	142.6
	5.1 (68)	0.7 (70)	0.2 (70)	0.1 (68)	3.3 (68)	12.5 (60)
	41-234	18-42	1.6-8.5	0.1-0.6	1-116	27-383
8	131.7	31.6	4.2	0.3	25.6	155.6
	5.8 (63)	0.7 (65)	0.2 (59)	0.1 (59)	3.7 (61)	15.6 (46)
	19-211	15-42	0.5-7.3	0.01-0.5	2-169	25-397
9	123.3	31.5	4.0	0.3	28.7	152.2
	6.2 (58)	0.7 (62)	0.2 (57)	0.1 (57)	4.3 (56)	14.6 (50)
	19-238	17-42	0.5-8.5	0.01-0.6	3-171	19-390
10	118.8	31.4	3.8	0.3	24.2	151.6
	6.3 (51)	0.7 (46)	0.2 (45)	0.1 (46)	3.3 (48)	16.6 (36)
	18-214	20-41	0.5-7.9	0.01-0.5	2-94	30-393

* B/A ratios are expressed in µmol/g; multiply by ~ 0.066 to convert to B/A molar ratios.

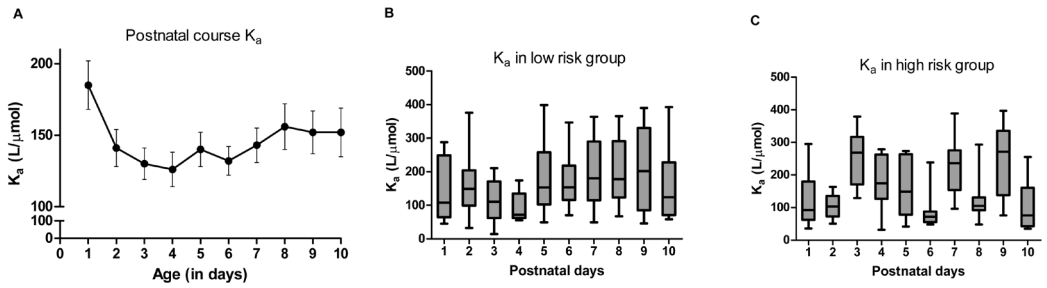

Figure 2. Variability of K_a during the first ten days of life.
A. Variability of K_a during the first ten days of life.
B. Postnatal course of K_a in ten low risk, clinical stable infants, defined as birth weight >1250 gram, and no presence of specified risk factors.
C. Postnatal course of K_a in ten high risk infants, defined as birth weight <1000 gram, and presence of two or more specified risk factors.
The median is marked by the horizontal line in the box. The boxes are limited by the 25th and 75th percentile. The whiskers (T) represent the lowest and highest K_a value measured on that day in one of the ten infants.

Figure 2C shows the variability of K_a in ten high risk infants, defined as BW <1000 gram, and presence of two or more specified risk factors. K_a values demonstrated large variability during the first ten days of life in preterm infants without and with additional bilirubin neurotoxicity risk factors.

Table 3 shows the K_a values for four predefined subgroups. K_a values were higher in preterm infants with prolonged GA, and with higher BW. We also evaluated the effect of PT. Five infants did not receive PT during the whole study-period. Most preterm infants received PT on postnatal days three and four. We found similar variability of K_a values in preterm infants not yet receiving PT, and in preterm infants under PT (data not shown).

Table 3. Binding affinity (K_a) between the B/A ratio and B_f in preterm infants divided into four subgroups according to birth weights and gestational ages.

Subgroup	n	K_a (range) in L/µmol
Birth weight <1250 g	43	97 (22-397)
Birth weight ≥1250 g	29	144 (14-399)
Gestational age <29 wks	34	88 (22-389)
Gestational age ≥29 wks	38	135 (14-399)

Estimation of possible B_f values – using equivalent mass action equations

Table 4 shows the range of possible B_f values if only TSB is known, and the range of possible B_f values if both TSB and Alb are known. We found a large range of B_f values using either method, although the range of B_f values by using the B/A ratio appeared somewhat smaller.

Table 4. The range of possible B_f values if only TSB was measured (known), and the range of possible B_f values if both TSB and Alb are known.

Day	B_f range (nM) if only TSB is known	B_f range (nM) if TSB and Alb are known
1	0.3-17	0.3-11
2	0.3-34	0.3-26
3	0.2-75	0.5-43
4	0.3-29	0.3-21
5	0.2-36	0.3-21
6	0.2-26	0.3-15
7	0.2-35	0.3-22
8	0.1-41	0.03-20
9	0.1-54	0.03-32
10	0.1-25	0.03-17

Estimation of possible B_f values – using multilevel analyses

In the Tables 5, 6, and 7 we present the multilevel models for the prediction of B_f by the B/A ratio, TSB, and Alb concentrations, respectively. No terms were included in the multilevel models in combination with GA of <29 weeks on day one, because there were no measurements that fulfilled these criteria (*i.e.* BW of >1250 gram and GA of <29 weeks on day one; n=2). B_f was significantly predicted by the B/A ratio in both BW and GA categories, except on day one (chi-square distribution; $p<0.05$). The same pattern was observed for TSB as for the B/A ratio, in that the TSB was a significant predictor for B_f from postnatal day two onwards ($p<0.05$). Alb was, compared to TSB and the B/A ratio, only a significant predictor of B_f on postnatal day one, based on the BW and GA categories we tested on the different days.

Table 5. Multilevel model for the prediction of free bilirubin (B_f) by B/A ratio.

Predictor term	Free bilirubin (B_f)		
	Beta (nmol/L)	T ratio	*p value*
B/A ratio x day 1 x BW >1250 g x GA > 29 wks	-1.54	0.36	.36
B/A ratio x day 2+3+4 x BW > 1250 g x GA > 29 wks	9.67	38.84	.000
B/A ratio x day >4 x BW >1250 g x GA > 29 wks	6.40	19.12	.000
B/A ratio x day 1 x BW < 1250 g x GA > 29 wks	8.98	1.14	.286
B/A ratio x day 2+3+4 x BW < 1250 g x GA > 29 wks	12.34	6.53	.011
B/A ratio x day > 4 x BW < 1250 g x GA > 29 wks	7.76	6.09	.014
B/A ratio x day 1 x BW < 1250 g x GA < 29 wks	6.09	2.19	.139
B/A ratio x day 2+3+4 x BW < 1250 g x GA < 29 wks	5.48	6.6	.010
B/A ratio x day > 4 x BW < 1250 g x GA < 29 wks	11.39	78.41	.000

B/A ratio: bilirubin/albumin ratio, BW: birth weight, GA: gestational age.

Table 6. Multilevel model for the prediction of free bilirubin (B_f) by TSB concentrations.

Predictor term	Free bilirubin (B_f)		
	Beta (nmol/L)	T ratio	*p value*
TSB x day 1 x BW >1250 g x GA > 29 wks	0.058	0.34	.37
TSB x day 2+3+4 x BW > 1250 g x GA > 29 wks	0.224	13.56	.000
TSB x day >4 x BW > 1250 g x GA >29 wks	0.138	6.9	.009
TSB x day 1 x BW< 1250 g x GA > 29 wks	0.222	1.09	.296
TSB x day 2+3+4 x BW < 1250 g x GA > 29 wks	0.313	5	.025
TSB x day >4 x BW< 1250 g x GA > 29 wks	0.289	9.71	.002
TSB x day 1 x BW < 1250 g x GA < 29 wks	0.243	1.6	.206
TSB x day 2+3+4 x BW < 1250 g x GA < 29 wks	0.268	14.91	.000
TSB x day > 4 x BW < 1250 g x GA < 29 wks	0.325	47.79	.000

TSB: Total serum bilirubin, BW: birth weight, GA: gestational age.

Table 7. Multilevel model for the prediction of free bilirubin (B_f) by Alb concentrations.

Predictor term	Free bilirubin (B_f)		
	Beta (nmol/L)	T ratio	*p value*
Alb x day 1 x BW>1250g x GA>29wks	2.24	1.82	.04
Alb x day 2+3+4 x BW>1250g x GA>29wks	0.562	2.92	.09
Alb x day >4 x BW>1250g x GA>29wks	-0.214	0.15	.7
Alb x day 1 x BW<1250g x GA>29wks	0.847	0.23	.63
Alb x day 2+3+4 x BW<1250g x GA>29wks	0.401	0.14	.71
Alb x day >4 x BW<1250g x GA>29wks	0.996	1.65	.2
Alb x day 1 x BW<1250g x GA<29wks	1.049	0.57	.45
Alb x day 2+3+4 x BW<1250g x GA<29wks	1.085	2.73	.1
Alb x day >4 x BW<1250g x GA<29wks	-1.182	5.81	.015

Alb: albumin, BW: birth weight, GA: gestational age.

DISCUSSION

This is the first study that provides serial data on the B/A ratio and B_f during the first ten days after birth in preterm infants. Interestingly, peak B_f concentrations were reached on the fourth postnatal day, one day after peak TSB concentrations and peak B/A ratios. We also examined the relationship between the B/A ratio and B_f, to test the hypothesis that the B/A ratio could serve as an alternative parameter for estimating B_f in preterm infants. Using equivalent mass action equations, the B_f range is smaller with the B/A ratio, than with TSB. However, the difference in favor of the B/A ratio does not seem to be clinically helpful; a rather broad range of B_f values for one single B/A ratio exists. In analogy, a multilevel model indicated that the B/A ratio was a significant predictor of B_f from postnatal day two onwards, but indicated also that the B/A ratio did not outperform TSB in this respect. In our opinion, this illustrated that the B/A ratio was neither an accurate alternative for B_f, nor a better surrogate parameter to estimate B_f than TSB.

Our results were in line with the low sensitivity and low positive predictive value of the B/A ratio for B_f in newborn infants ≥35 weeks.[14] The range of B/A ratios of 0.30 to 11.8 μmol/g (0.02 to 0.78 molar ratios) reported in the latter study was similar to the range of the B/A ratios in our study, whereas their mean binding affinities were somewhat lower than ours, *i.e.* approximately 80 to 100 L/μmol.[14]

In our study population binding affinity was the lowest in preterm infants with a GA of <29 weeks and a BW of <1250 gram, but with a relatively large interindividual variation. Direct comparison of the B_f concentrations in our study with literature data is complex. In contrast to national and international standardizations for TSB and Alb measurements[15], no "gold standard" is available for B_f. On the contrary, several instruments are available, each with a different analytical methodology. As a result, B_f values measured by different research groups varies considerably, as is clear from Table 8. A manual method evaluated and approved by the FDA is currently commercially available (Arrows Co. LTD, Osaka, Japan). It is, however, not readily available outside Japan. The peroxidase method that we used measures both TSB and B_f, and is the most commonly used method.[16]

Prerequisite for the Arrows method is a 42-fold sample dilution before analysis. This may, at least in part, underlie the differences in B_f concentrations between our study and others, because sample dilution has been shown to intrinsically alter bilirubin-albumin binding. In addition, the number of peroxidase concentrations (one versus multiple) is essential. Minimal sample dilution, and the use of more than one peroxidase concentration, as we did in our B_f measurement operating procedure, is advocated to improve the accuracy of the B_f measurement.[17]

Table 8. Peak B_f concentrations in preterm and full term infants assessed with different methodologies.

Methodology	N	Gestational age (wks)	Birth weight (g)	Peak B_f (nmol/L)
Arrows UB Analyzer Funato *et al.* (1994)	37	38.8 (±1.0)	3137 (±396)	19.3 (±7.5)
Arrows UB Analyzer UA-1 Amin *et al.* (2004)	20	27.7 [24-32]	-	6.8 (±1.1)
UB-Analyzer UA-2 Lee et al. (2009)	86	28.3 (±3.1)	1075 (±298)	8 [1.4-30.6]
Arrows UB Analyzer Ahlfors *et al.* (2009)	175	34 (±5) [24-41]	2246 (±1104) [406-4727]	15.9 (±12)
Arrows UB Analyzer Sato *et al.* (2012)	209	39 [35-41]	2854 [1592-4076]	0.6 [0-20.5]
Zone Fluidics system (Global Flopro) Schreuder *et al.*	72	29.2 (±1.6)	1226 (±288)	49 (±5)

Data are expressed as mean (± SD) and/or median [range] values.

In addition to these analytical issues, B_f concentrations may also be affected by patient-related factors. Oh *et al.* found that clinical status assessed on the fifth postnatal day influenced B_f concentrations.[19]

In a retrospective analysis of infants with extremely low BWs (ranging from 401 to 1000 gram), the infants with sepsis had higher B_f values than those without sepsis with similar TSB concentrations.[19] Probably, sepsis impairs K_a, comparable to the bilirubin-displacing effect of many drugs and endogenous substances.[20]

Our data clearly demonstrated that K_a is also variable in clinically stable infants during the first ten days of life. In fact, we found that the K_a value ranged between 14 and 399, which may be the most important explanation why using the B/A ratio does not add much advantage in estimating B_f compared to TSB alone. In general, TSB and B_f peak concentrations did not necessarily occur concomitantly. It is tempting to speculate that the delayed B_f peak was due to, as yet unrevealed, patient-related factors that influenced the binding of bilirubin to albumin. Although we did find a moderate effect of GA and BW on absolute K_a values, broad ranges of K_a values existed. Phototherapy did not affect variability of K_a.

The effect of clinical pathophysiological factors or substances that affect the binding of bilirubin to albumin requires further study in helping us understand the vascular factors enhancing the risk of BE, and how bilirubin-albumin binding may be influenced positively in infants with imminent BE. The clinical applicability of the B/A ratio in predicting B_f in individual patients seemed limited; the

B/A ratios should not replace B_f measurements. Rather than using the B/A ratios in addition to TSB, the development of easily applicable B_f measurements may allow for more accurate estimation of brain bilirubin exposure in preterm infants. There is an urgent need to establish at least the "normal" values for B_f in various populations, and to document B_f concentrations in (preterm) newborn infants with suspected BE. This may ultimately help to individualize the clinical approach to jaundiced newborns. The tendency towards increased mortality in sick, extremely low BW infants exposed to PT, stresses the importance of our recommendation.[21,22]

CONCLUSION

Peak B_f concentrations in preterm infants of <32 weeks' of gestation did not necessarily occur simultaneously with peak TSB concentrations. Our data show that there is little added clinical advantage to using the B/A ratio, as compared to TSB alone, for estimating B_f. The large variability of bilirubin-albumin binding needs further study.

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Chapter 7

GENERAL DISCUSSION, CONCLUSIONS, AND FUTURE PERSPECTIVES

7.1. INTRODUCTION

This thesis focuses on new diagnostic and therapeutic approaches for severe unconjugated hyperbilirubinemia. Severe unconjugated hyperbilirubinemia, as can occur in Crigler-Najjar disease and neonatal jaundice, carries the risk of neurotoxicity.

At the moment, treatment criteria for hyperbilirubinemia are based on total plasma bilirubin concentrations. It is known, however, that plasma bilirubin concentrations correlate poorly with the occurrence of brain damage in individual patients.[1] The reason for this poor correlation lies in the inability of protein-bound bilirubin (>99% of total plasma bilirubin) to leave the circulation.[1-4] The free bilirubin concentration (B_f), the fraction of bilirubin not bound to plasma proteins, has been suggested to more closely predict the occurrence of brain damage. Only B_f can translocate across the blood-brain barrier, and can cause brain damage. According to the "free bilirubin hypothesis"[5], the total plasma bilirubin and B_f concentrations establish a steady state across the blood-brain barrier and the neuronal cell membrane, with B_f in the interstitial fluid and B_f in the neuron. At nontoxic bilirubin levels, the B_f in these compartments is also in a steady state with bilirubin bound to albumin, membranes, and cell organelles. Bilirubin toxicity occurs if the B_f reaches levels where it becomes insoluble, and/or membranes and organelles are sufficiently saturated to disrupt function.[6] The risk of bilirubin toxicity will therefore be proportional to the plasma B_f, but modulated by the other risk factors present such as low gestational age, and metabolic integrity (*e.g.* acidosis).[7]

Cornerstones in the treatment for hyperbilirubinemia are phototherapy (PT) and exchange transfusion (ET). Phototherapy is generally successful in decreasing plasma bilirubin concentrations, but sometimes not sufficient to reach non-toxic levels. Exchange transfusion can be seen as a "rescue treatment", when bilirubin concentrations are extremely high or when PT fails to prevent a further increase in plasma bilirubin concentrations, and thereby the risk of brain damage.

For the experiments in this thesis we used the Gunn rat model, the well-established animal model for severe unconjugated hyperbilirubinemia.[8-10] Gunn rats have a life-long unconjugated hyperbilirubinemia due to a spontaneous mutation in the hepatic enzyme uridine-diphosphoglucuronosyltransferase: UGT1A1.[11-14]

7.2. THE CLINICAL SITUATION

7.2.1. The bilirubin/albumin ratio in preterm infants

Neonatal unconjugated hyperbilirubinemia occurs in almost all preterm infants and may result in bilirubin encephalopathy. As mentioned above, it has been demonstrated repeatedly, that B_f predicts bilirubin-induced brain damage better than total serum bilirubin (TSB) concentrations.[3,15,16] However, current treatment strategies are still based on TSB concentrations[17], and B_f is not routinely incorporated in clinical practice. The main reason for this lies in the inaccuracy of the commercially available B_f test method, most notably caused by a 42-fold sample dilution that alters bilirubin-albumin binding.[18] The bilirubin/albumin (B/A) ratio was put forward as an alternative parameter for estimating B_f.[19] The B/A ratio can be obtained easily, because TSB and albumin are both measured using routine laboratory techniques. Information on the postnatal course of the B/A ratio and of B_f in preterm infants, and how they are interrelated, is limited. In chapter 6 we described the first study providing serial data on the postnatal course of the B/A ratio and B_f during the first ten days after birth in preterm infants. We hypothesized that the B/A ratio could serve as an alternative parameter for estimating B_f in preterm infants. By using equivalent mass action equations we showed that the B_f range is smaller with the B/A ratio, than with TSB. However, the difference in favor of the B/A ratio does not seem to be clinically helpful; since for one single B/A ratio a broad range of B_f values exists. In parallel, a multilevel model indicated that the B/A ratio was a significant predictor of B_f from postnatal day two onwards, but the B/A ratio did not outperform TSB in this respect. In our opinion, our data show that there is little added clinical advantage to using the B/A ratio, as compared to TSB alone, for estimating B_f. We recommend to make B_f measurements routinely available to individualize the clinical approach of jaundiced preterm infants.

The impact of bilirubin-albumin binding on the relationship between the TSB and the amount of bilirubin that has accumulated in the brain (*i.e.* the risk of kernicterus) can be demonstrated by considering two identical newborns, except for two different albumin concentrations, for example of 4 g/dL and 2 g/dL.[20] Given equal bilirubin production rates, if the baby with the lower albumin reaches a TSB of 15 mg/dL, and for example the B_f levels are 3 µg/dL, then by mass action equation the baby with the higher albumin would have a B_f of 1.5 µg/dL. Since the albumin and B_f levels are different, the brain bilirubin levels, and risks of kernicterus are different for both newborns despite equal TSB concentrations. Therefore, it is worth considering albumin administration in infants with low plasma albumin levels.

To obtain better insights in the mechanisms by which bilirubin causes brain damage and by which treatments counteract this, we performed several animal experiments.

7.3. EFFECTS OF HYPOBILIRUBINEMIC TREATMENTS ON BRAIN BILIRUBIN LEVELS IN RATS

7.3.1. Phototherapy and albumin prevent bilirubin accumulation in the brain

As stated above, B_f plays a key role in the pathogenesis of bilirubin-induced brain damage. We hypothesized that administration of albumin could decrease the translocation of B_f to the brain, and thus prevent bilirubin-induced brain damage, by providing more binding sites for bilirubin and thus likely decreasing B_f.

We evaluated the effect of human serum albumin (HSA) treatment on brain bilirubin levels

of phototherapy-treated Gunn rats (Chapter 2). Our experiments were performed in either permanently or acutely (*i.e.* hemolytic) jaundiced Gunn rats, which served as a model for Crigler-Najjar disease or neonatal hemolytic jaundice, respectively.[21] In non-hemolytic Gunn-rats, long-term phototherapy decreased plasma unconjugated bilirubin (UCB), plasma B_f and brain bilirubin concentrations. Adjunct HSA treatment increased PT efficacy by ~35%. In hemolytic Gunn rats, PT+HSA similarly decreased plasma B_f concentrations, and the combination even completely prevented bilirubin accumulation in the brain. A remarkable finding was the inability of PT alone, *i.e.* the conventional present treatment, to protect these animals from bilirubin accumulation within their brains. Taken together, our data showed that HSA provides a synergetic effect to PT in Gunn rats. We speculate that HSA and PT act *in tandem*. First HSA decreases B_f within the plasma, which promotes a bilirubin shift from the brain into the blood. Phototherapy then converts this bilirubin into photo-isomers which can readily be excreted within the bile.

Interestingly, HSA treatment has been used in studies with severely jaundiced neonates.[22-25] These studies compared the effect of albumin administration with that of ET[22,23] or of intensive PT.[24,25] Some studies found a beneficial effect of albumin administration, *e.g.* a more profound decrease in plasma TSB concentrations[22,25], while others did not observe this advantageous effect.[23,24] The discrepancy between the different studies may originate from the inability to measure brain bilirubin levels in humans, and from the aforementioned inaccuracy of the commercially available B_f test.

We consequently feel that large-scale clinical trials are the next step towards the routine application of HSA in a clinical setting. These trials should incorporate B_f measurements and, ideally, a functional marker of brain function, such as auditory brain stem response measurements. These measurements would allow noninvasive monitoring of bilirubin-induced brain damage, and have been well described in neonates.[21,26,27] As mentioned above, there is still no reliable method to analyze B_f for acute clinical situations. However, B_f values can be measured afterwards, in a more experimental setting, and can tell us something about the neurotoxic risk.

7.3.2. Exchange transfusion is the most effective acute treatment option

Presently, the standard treatment for hyperbilirubinemia is PT. Phototherapy is generally effective, but in some neonates the plasma bilirubin concentrations nevertheless become dangerously high, or continue to rise, for example during ongoing hemolysis. In these patients PT alone might fail to prevent bilirubin-induced brain damage, and for these patients ET is indicated. Exchange transfusion is considered as a "rescue treatment", based on its invasiveness and related risks. The mortality rate from the procedure is approximately 0.3-2.0%. Significant morbidity is associated with 5-12% of ETs.[28-30] It has remained unclear whether ET could successfully be replaced by other, equally effective treatment options but with lower morbidity and mortality. Albumin administration might be a good treatment modality. For example, in chapter 2, we found in Gunn rats that adjunct HSA can increase the efficacy of PT; it decreased plasma B_f concentrations and

brain bilirubin levels by ~90% and ~70%, respectively.[31] Studies to replace ET, to improve ET efficacy and/or to minimize its risks have been hampered by the contemporary low application rate of ET in humans, and by the lack of an appropriate *in vivo* model system. The absence of an appropriate animal model has also prevented the determination of the efficacy of adjunct or alternative treatment options such as albumin administration.

In chapter 3 we describe the successful use of Gunn rats as a tool to optimize a model for ET during unconjugated hyperbilirubinemia. We also showed that this Gunn rat-ET model might be very valuable to evaluate the effect of modulating ET procedures and techniques, and to compare its efficacy in combination with other treatments to prevent brain damage during acute severe hyperbilirubinemia. Additionally, we concluded that ET is the most effective treatment option with respect to the acute treatment of hyperbilirubinemia. For the long-term treatment after ET, the combination of PT+HSA is most effective in maintaining the hypobilirubinemic effect.

In our study we used a PT intensity of 18 $\mu W/cm^2/nm$, which can be seen as "normal" PT. However, another way to decrease bilirubin quickly is with intensive PT, *i.e.* a PT intensity of more than 30 $\mu W/cm^2/nm$. It is known, that a dose-response relationship exists between PT and the decrease in TSB in infants (gestational age ≥33 weeks) with hyperbilirubinemia.[32] In 24h, a decrease in TSB of 34% can be achieved with a median light irradiance of 26 (range 24-28) $\mu W/cm^2/nm$. For a median light irradiance of 36 (range 36-39) $\mu W/cm^2/nm$ this is a decrease of 42% in TSB, and for a median light irradiance of 49 (range 47-51) $\mu W/cm^2/nm$ a decrease of 47% in TSB was shown.[32] In our ET-model in rats we showed a decrease in plasma TSB of 44%, and 88% in plasma B_f concentrations in 1h. Although we are now comparing an animal study with a clinical study, we still think that an ET is the most effective acute treatment option. For intensive PT you need 24h of extremely high irradiance levels to provide the same hypobilirubinemic effect as after 1h following ET. For clinical conditions in which a rapid decline of toxic bilirubin levels is warranted, ET seems therefore a more appropriate strategy.

7.3.3. Albumin treatment is functionally neuroprotective in Gunn rat pups

Since we were interested in neonatal jaundice, we considered it important to also perform treatment experiments in Gunn rat pups. We expected Gunn rat pups to resemble the clinical situation in neonates more closely than adult Gunn rats. In chapter 4 we evaluated the effects of albumin treatment on brainstem auditory evoked potentials (BAEPs). Brainstem auditory evoked potentials (or auditory brainstem responses, ABRs) assess neural transmission between the auditory nerve and auditory brainstem structures.[33] This method is based on the vulnerability of the auditory system to hyperbilirubinemia.

We used two Gunn rat pup models of acute hyperbilirubinemia mimicking severe neonatal hyperbilirubinemia: one due to hemolysis, and the other one based on drug-induced displacement of bilirubin from albumin. For the hemolysis model we used phenylhydrazine to induce hemolysis. For the bilirubin-albumin displacement model we used sulfadimethoxine, which is a compound

that competes with bilirubin for binding to serum albumin, and results in accumulation of B_f in lipophilic tissues, including the brain.[34-37] To assess bilirubin neurotoxicity we used the validated BAEP method, and compared relevant parameters between albumin-treated and control rat pups (peak latency values and interwave-interval (IWI) between peak I and peak II). An increased IWI I-II is a reflection of acute neurotoxicity.[33] We show that albumin treatment is neuroprotective in acute hyperbilirubinemia in Gunn rat pups, irrespective of its nature, *i.e.* induction by hemolysis or by bilirubin-albumin displacement.

One of the clinical challenges with hyperbilirubinemia is, especially in premature neonates, to determine when bilirubin exposure becomes toxic to the brain and auditory system. The use of TSB concentrations and the use of automated auditory brainstem response (AABR) are helpful, but they remain relatively crude indications of neurotoxicity. It would seem more useful to have information of B_f, TSB and bilirubin binding, coupled with diagnostic ABRs, which are very sensitive to subtle bilirubin neurotoxicity. Performing ABRs in the nursery, especially in premature infants, can be challenging but is not impossible. Some of these challenges have been solved by the AABR, which unfortunately is not expected to be sensitive to subtle abnormalities of ABR that would occur at the onset of neurotoxicity.[38]

The normal BAEPs (functional results) compared to the increased UCB brain levels (biochemical results) in the experiments of chapter 4, show the importance of functional diagnostic tests, particularly in the field of unconjugated (free) bilirubin. This discrepancy between BEAPs and UCB brain levels has never been described before. On the other hand, we also evaluated B_f and TSB concentrations in our study, and studies about the importance of these other biochemical results compared to ABRs are known.

An observational study of 191 newborns showed that an abnormal AABR is associated with elevated B_f concentrations, but not with TSB concentrations alone.[26]

Another study in 44 jaundiced newborns (gestational age ≥34 weeks), showed that an abnormal ABR is associated with increased B_f concentrations, but not with increased TSB concentrations.[39]

The prevalence of bilirubin neurotoxicity as a cause of audiological dysfunction may be underestimated, when TSB alone is used to assess the severity of newborn jaundice.[26]

Because B_f concentrations are more closely correlated with bilirubin neurotoxicity than are TSB concentrations, bilateral refer AABR results for jaundiced newborns may indicate increased risk of bilirubin neurotoxicity, in addition to the possibility of congenital deafness.[39]

The beneficial, functional results of our study favor the clinical potency of albumin treatment to prevent or mitigate neurotoxicity due to severe neonatal hyperbilirubinemia.

7.4. PREVENTION OF SEVERE NEONATAL JAUNDICE IN GUNN RAT PUPS

7.4.1. Polyethylene glycol and ursodeoxycholic acid prevent neonatal jaundice

Once jaundice is present several therapies, like PT and ET, are available. Unfortunately, however, *preventive* strategies against neonatal jaundice are presently limited. That is why we did not only evaluate treatment options for hyperbilirubinemia in this thesis, but also described a study about prevention of hyperbilirubinemia.

Worldwide there are large newborn populations for whom PT is not available as a therapeutic modality, and in these frequently resource limited countries prevention can be of great importance. But also in the Western world, it would be beneficial to find a cheap and safe preventive strategy for severe jaundice, which can be for example given at home. In chapter 5 we tested in a Gunn rat pup model the hypothesis that polyethylene glycol (PEG) and ursodeoxycholic acid (UDCA) can be preventive strategies for neonatal jaundice. We chose PEG and UDCA as preventive options, since it is known that fecal excretion of bilirubin constitutes the main excretory pathway of bilirubin for the body. We previously demonstrated in adult rats that unconjugated hyperbilirubinemia could be treated by PEG or UDCA, through enhancing the fecal UCB excretion. The preventive capacity of this strategy for neonatal jaundice, however, was unknown. We found that oral administration of either PEG or UDCA prevented the hyperbilirubinemic peak in Gunn rat pups, probably by enhancing its fecal transport. We can only speculate that the preventive effect of PEG and UDCA is due to the same mechanism shown in adult Gunn rats, namely fecal excretion of UCB. We were not able to measure the fecal bilirubin disposal in pups.

One other method is known to interdict with the development of severe jaundice, namely via heme oxygenase inhibitors, *i.e.* porphyrins. Presently, the only synthetic inhibitor that is approved by the Food and Drug Administration for clinical use is Sn-mesoporphyrin (SnMP). Several studies by Kappas *et al.* showed that SnMP in infants is safe, and that the treatment is an effective prevention option for hyperbilirubinemia.[40-42] However, porphyrins are not routinely used in the clinical setting. The reason for this is that there can be adverse metabolic consequences of SnMP therapy, and that studies about cost-effectiveness of SnMP are awaited. In the case of PEG and UDCA the availability in the clinic is different. Polyethylene glycol and UDCA are already used in the clinical situation, for example PEG in children with constipation[43], and UDCA in pediatric patients with cystic fibrosis liver disease.[44] In our study, the preventive dosage of PEG given to rats is several fold lower compared to the treatment in infants. We think that PEG might be a good candidate for prevention of hyperbilirubinemia in the clinical situation, since PEG treatment for constipation requires higher dosages, and those higher dosages are already well-tolerated by children.[45,46] Numerous clinical trials with PEG have shown an absence of serious side effects.[47] The reverse, our preventive dosage of UDCA in rats is higher than the treatment dosage used in infants. Therefore, we like to underline that the present results of UDCA should be considered as a "proof-of-principle". We did not evaluate an optimal dose for rat pups, but used a similar dosage of UDCA that had been

proven effective in adult Gunn rats.
Our study underlines the need to critically evaluate the use of PEG and UDCA as preventive treatment option in jaundiced newborns in clinical trials. These studies will, hopefully, allow to further decrease the incidence of bilirubin-induced brain damage worldwide in the future.

7.5. CONCLUSIONS AND FUTURE PERSPECTIVES

In this thesis we demonstrated that albumin administration combined with PT and/or ET increases the efficacy of both acute and chronic treatment for unconjugated hyperbilirubinemia in Gunn rats. In addition we showed that albumin treatment has beneficial, functional results (*i.e.* normal BAEPs) in acute hyperbilirubinemic Gunn rat pups. Also, we showed two strategies to prevent the hyperbilirubinemic peak in Gunn rat pups. Finally, we demonstrated that the B/A ratio is a significant predictor of B_f in preterm infants, but the B/A ratio does not outperform TSB in this respect.

The effect of albumin treatment was studied in Gunn rats, the well-established animal model for unconjugated hyperbilirubinemia. The use of an animal model enabled us to study the therapeutic possibilities, and the underlying mechanism of this new treatment. In the clinical setting, albumin treatment is already used in combination with an ET in severely jaundiced neonates, when donor blood is not immediately available[23], but this approach has been disputed.[22,23]
Consequently, a logical step would be to determine the effects of albumin treatment in clinical trials. The first step would be to evaluate, if we are able to decrease the need of an ET with help of HSA treatment. In this case, HSA can also be seen as a rescue treatment, prior to the more invasive treatment option in the form of an ET.
The second step would be to perform a trial with HSA and PT in patients with Crigler-Najjar disease. These patients already require life-long PT, and would thus benefit directly from HSA in case of exacerbations. Such a study would consist of daily albumin administration combined with PT during the exacerbation period. The study-population would consist of Crigler-Najjar patients with increased hyperbilirubinemia due to intercurrent illness, surgery or noncompliance. To assess the effects of this treatment combination, it is useful to measure plasma UCB and B_f concentrations, and time spent under PT. If HSA treatment proved to be effective, not only the time spent under PT could be decreased dramatically, also the chance on brain-damage will be lowered. However, clinical trials in Crigler-Najjar patients can be difficult, due to the low prevalence of this disease.[48] It seems therefore needed to design a multi-center and probably "multi-country" study.
The next step would be a clinical trial in a more vulnerable patient-group: neonates. It will be interesting to evaluate the role of HSA administration in combination with PT, but also with ET. Furthermore, it is known that after ET, a rebound of plasma bilirubin concentrations of about 50% occurs.[49,50] It would be attractive to evaluate the role of albumin in the long-term management after

ET. In order to study this efficacy, it is necessary to evaluate reliable predictors for bilirubin-induced neurological damage. We would suggest using B_f and auditory brain stem response measurements in this case. These markers are essential, since it is obviously impossible to measure UCB in the brain of human neonates.
The final step would be prevention instead of treatment. We think that a clinical trial would be useful to see if PEG and UDCA are also preventive strategies for hyperbilirubinemia in infants. Next, it would be useful to carry out a cost-benefit analysis to determine the relative economics of phototherapy-treatment versus prevention with oral treatment with PEG or UDCA. We believe that prevention is always better than cure, but prevention should be cost-effective and also feasible for the patient.

Moving forward from bench to bedside by continuing our research in a more clinical setting is an important step. It makes it possible to evaluate the potential of HSA as an alternative or additional treatment option for severely hyperbilirubinemic patients. However, we should simultaneously continue the use of *in vitro* and *in vivo* studies, to increase our knowledge of the mechanisms underlying bilirubin neurotoxicity, and the possibilities to interfere with this mechanism by means of hypobilirubinemic treatments. Understanding the mechanisms will create a stable underground to design and develop new treatment modalities for hyperbilirubinemia, and decrease the incidence of bilirubin toxicity in patients.

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Appendices

SUMMARY

In this thesis we developed and refined diagnostic and treatment strategies for severe unconjugated hyperbilirubinemia. Our studies were performed in the Gunn rat model and in human (preterm) neonates. Severe unconjugated hyperbilirubinemia, as occurs in Crigler-Najjar disease and in neonatal jaundice, is associated with brain damage and even death. At the moment, standard treatment principally consists of phototherapy (PT). Although generally effective, PT has some disadvantages. The lifelong PT needed for patients with Crigler-Najjar disease, which have a genetic liver enzyme defect that leads to inherited jaundice, may last up to 16h per day, but fails to prevent brain damage in ~25% of these patients. Phototherapy for neonates, although mostly safe and effective, sometimes fails to achieve a sufficient decrease in plasma bilirubin concentrations. Eventually, this may also lead to permanent brain damage in neonates.

The fact that we still have patients who develop brain damage, underlines the need for alternative or additional treatment options. Currently, treatment for hyperbilirubinemia is initiated and stopped on total plasma bilirubin concentrations (UCB). However, it is well known that UCB correlates poorly with the occurrence of brain damage in patients. The reason for this poor correlation is, that not the total amount of UCB in plasma, but only free bilirubin (B_f), the small fraction (<1%) of bilirubin not bound to plasma proteins, can translocate across the blood-brain barrier, and can cause brain damage. B_f plays a major role in the pathogenesis of bilirubin-induced neurotoxicity. Theoretically, decreasing the B_f fraction in plasma, for instance by increasing its binding to albumin, would thus be expected to decrease brain bilirubin levels. In **chapter 2** we investigated the effect of PT and PT plus albumin administration on plasma B_f, and on brain bilirubin levels during permanent (*i.e.* Crigler-Najjar disease) and acute (*i.e.* neonatal hemolytic jaundice) hyperbilirubinemia in Gunn rats. Our results showed that albumin provides a synergistic effect together with PT in Gunn rats. In permanently jaundiced Gunn rats, long-term PT combined with albumin treatment decreased plasma UCB, plasma B_f, and brain bilirubin concentrations profoundly, compared to PT alone. In acutely jaundiced Gunn rats, PT plus albumin not only decreased plasma B_f concentrations, but also completely prevented bilirubin accumulation in the brain. Interestingly, PT alone failed to prevent bilirubin accumulation in the brains of the acutely jaundiced rats. Taken together, our results indicate that indeed treating hyperbilirubinemia with albumin administration could lower B_f and brain bilirubin content, and is likely preventive for bilirubin neurotoxicity. We think that these results provide the basis for a randomized controlled clinical trial, to evaluate the use of adjunct albumin treatment in combination with PT in as well Crigler-Najjar patients as jaundiced neonates.

Based on these successful and promising effects of albumin treatment, we continued our experiments with albumin. In **chapter 3** we studied the hypobilirubinemic treatment effects of the

combination of exchange transfusion (ET), PT and/or albumin administration. An ET is considered a "rescue treatment" when bilirubin concentrations are extremely high, or fail to respond to PT. However, an ET has considerable risks. We hypothesized that the combination of PT and albumin might even be more effective than an ET. First, we successfully optimized the model for ET in Gunn rats during unconjugated hyperbilirubinemia. Our results showed that this Gunn rat-ET model might be very valuable to compare the efficacy of ETs to other treatments, or treatment combinations, to prevent brain damage during acute severe hyperbilirubinemia.
We showed that ET is the most effective treatment option in the acute situation. After 1h of treatment, ET showed a significantly stronger decrease in plasma UCB and B_f compared to the combination of PT plus albumin. As an acute treatment option, the combination of an ET with PT and/or albumin did not show a significantly stronger hypobilirubinemic effect than ET alone. As follow up treatment after ET, the combination of PT with albumin is most effective in maintaining this hypobilirubinemic effect over 48h. In our opinion, the availability of this newly developed Gunn rat-ET model could be very helpful to further optimize the treatment for acute, potentially neurotoxic hyperbilirubinemia.

In chapter 2 and 3 we evaluated the effect of albumin in combination with different treatment options on brain bilirubin levels. In addition to the biochemical results, we felt the importance to address the effects of hyperbilirubinemia and its treatments on a more functional parameter of brain toxicity. In **chapter 4** we not only made the step from adult rats to Gunn rat pups, but we also determined for the first time the effects of albumin treatment on a parameter for brain function, and related this to the brain bilirubin levels.
Bilirubin neurotoxicity can be assessed by brainstem auditory evoked potentials (BAEPs), based on the vulnerability of the auditory system to hyperbilirubinemia. BAEPs are a functional diagnostic test, and assess neural transmission between the auditory nerve and auditory brainstem structures. In our experiments we induced acute hyperbilirubinemia *via* two different strategies, namely through hemolysis and through bilirubin-albumin displacement. We showed that albumin treatment was neuroprotective in both acute hyperbilirubinemic models in Gunn rat pups. We also found a discrepancy between BAEPs (functional results) and UCB brain levels (biochemical results), which showed the importance of functional diagnostic tests, particularly in the field of unconjugated (free) bilirubin. Taken together, our beneficial, functional results favor the clinical potency of albumin treatment to prevent or mitigate neurotoxicity due to severe (neonatal) hyperbilirubinemia.

Once jaundice is present, several therapies are available, such as phototherapy and/or an exchange transfusion, as shown in chapter 2 and 3. Unfortunately, however, preventive strategies against neonatal jaundice are presently limited. Fecal excretion of bilirubin or of its degradation products constitutes the main excretory pathway of bilirubin for the body. We previously demonstrated that

unconjugated hyperbilirubinemia could be treated in adult Gunn rats by enhancing the fecal UCB excretion with polyethylene glycol (PEG) or ursodeoxycholic acid (UDCA). The preventive capacity of this strategy for neonatal jaundice is still unknown. In **chapter 5** we determined whether PEG or UDCA treatment could prevent unconjugated hyperbilirubinemia in Gunn rat pups, a model for neonatal jaundice. We daily measured transcutaneous bilirubin (TcB) concentrations, a method which we first validated, to follow bilirubin concentrations in pups from postnatal day 1 till day 21. At postnatal day 7 we started daily treatment with either saline (control), PEG, or UDCA *via* oral gavage. We found that the natural course of the hyperbilirubinemic TcB peak occurred on postnatal day 15 till 18. PEG and UDCA significantly decreased this hyperbilirubinemic TcB peak compared with controls. In chapter 5 we provide a proof of concept in a rat pup model for severe neonatal jaundice, that treatment with either PEG or UDCA can be a preventive strategy for neonatal jaundice. The prevention of bilirubin accumulation in plasma is probably due to the induction of the fecal disposal of bilirubin. The present results open the perspective for a preventive strategy for neonatal jaundice in (preterm) neonates.

Finally, we made the step from bench to bed. Neonatal unconjugated hyperbilirubinemia occurs in almost all preterm infants and may result in bilirubin encephalopathy. Current treatment strategies for severe hyperbilirubinemia are based on total serum bilirubin (TSB) concentrations and not on B_f concentrations. As stated above, B_f predicts bilirubin-induced brain damage better than TSB concentrations. Nevertheless, B_f has not been routinely incorporated in clinical practice, related to analytical difficulties to provide reliable results on B_f concentrations rapidly. The bilirubin/albumin (B/A) ratio was put forward as an alternative parameter for estimating B_f. In **chapter 6** we described for the first time serial data on the postnatal course of the B/A ratio and of B_f during the first ten days after birth in preterm infants. We found that peak B_f concentrations were reached on the fourth postnatal day, one day after peak TSB concentrations, and peak B/A ratios were reached. Using equivalent mass action equations, we showed that the B_f range is smaller with the B/A ratio, than with TSB alone. However, the difference in favor of the B/A ratio does not seem to be clinically helpful; a rather broad range of B_f values for one single B/A ratio exists. Next, by using a multilevel model we demonstrated that the B/A ratio was a significant predictor of B_f from postnatal day two onwards, but we also observed that the B/A ratio did not outperform TSB in this respect. We concluded that there is little added advantage by using the B/A ratio compared to TSB alone for estimating B_f. We recommend to make B_f measurements routinely available to individualize the clinical approach of jaundiced preterm infants.

In the final chapter of this thesis, **chapter 7**, we place our results in an experimental and clinical framework, and discuss future perspectives.

In this thesis we have developed and improved diagnostic and treatment strategies for unconjugated hyperbilirubinemia. The new strategies may ultimately serve as an alternative or additional option for routine treatment, and may prevent bilirubin-induced brain damage in hyperbilirubinemic patients. Accordingly, we think that our results can be used as a starting point for the development of clinical trials in Crigler-Najjar patients and in severely jaundiced neonates.

NEDERLANDSE SAMENVATTING

Bilirubine is een gele kleurstof in ons bloedplasma. Bij gezonde mensen is bilirubine in lage concentraties in het plasma aanwezig en werkt het als een antioxidant. Er zijn echter ook ziekten en situaties waarbij hoge bilirubineconcentraties ontstaan, die zeer schadelijk zijn voor onze lichaamscellen. Vooral hersencellen zijn gevoelig voor toxiciteit van bilirubine, waardoor ophoping van bilirubine in ons lichaam kan leiden tot permanente schade in ons brein. In dit proefschrift hebben wij in zowel een diermodel als bij pasgeboren baby's nieuwe diagnostische en therapeutische mogelijkheden onderzocht om hersenschade te voorkomen.

Bilirubine wordt gevormd door de afbraak van rode bloedcellen. Deze afbraak, en dus de productie van bilirubine, is een continu proces. De uitscheiding van bilirubine vindt plaats via de lever. De lever neemt bilirubine op uit de bloedbaan, verandert (conjugeert) het en transporteert het naar de gal. Via de galwegen wordt het geelgekleurde molecuul dan uitgescheiden in de darm, waarna het van kleur verandert en ons lichaam als bruine kleurstof verlaat via de ontlasting. De uitscheiding van bilirubine via de galwegen en de ontlasting moet uiteraard even groot zijn als de aanmaak van bilirubine uit de rode bloedcellen om te voorkomen dat de bilirubinespiegel stijgt. Wanneer er meer bilirubine wordt aangemaakt dan uitgescheiden, zal de gele kleurstof zich ophopen in het plasma (hyperbilirubinemie), de weefsels en de organen. De ophoping van bilirubine in de weefsels zorgt voor een kenmerkende gele verkleuring van de huid en het oogwit, ook wel geelzucht genoemd. Ernstige ongeconjugeerde hyperbilirubinemie kan leiden tot hersenschade, omdat het toxische bilirubine zich kan ophopen in ons brein. Deze hersenschade komt hoofdzakelijk voor bij patiënten met de ziekte van Crigler-Najjar en in bepaalde gevallen bij pasgeborenen.

Patiënten met Crigler-Najjar hebben, door een erfelijk defect, een niet (type I) of nauwelijks (type II) werkzame variant van het leverenzym UDP-glucuronosyltransferase (UGT1A1). Dit leverenzym is van belang voor de uitscheiding van bilirubine, omdat het bilirubine koppelt aan twee suikergroepen. Deze koppeling, ook wel conjugatie genoemd, is nodig om bilirubine op een efficiënte manier uit te scheiden vanuit de lever naar de gal. Aangezien de ziekte van Crigler-Najjar berust op een genetisch defect, lijden deze patiënten aan een levenslange ophoping van ongeconjugeerd (niet gekoppeld, schadelijk) bilirubine.

Ook pasgeborenen ontwikkelen vaak een ongeconjugeerde hyperbilirubinemie, maar deze is slechts enkele dagen aanwezig en is doorgaans niet schadelijk. De oorzaak van deze neonatale geelzucht ligt in een tijdelijk verhoogde afbraak van rode bloedcellen, in combinatie met een nog lage activiteit van het eerder genoemde leverenzym UGT1A1. In sommige gevallen is er toch behandeling nodig om hersenschade te voorkomen. Vaak is dit bij kinderen met een bijkomend probleem, zoals bijvoorbeeld bij bloedgroep-incompatibiliteit tussen moeder en kind. In dat geval zorgen antistoffen van de moeder voor een massale afbraak van rode bloedcellen bij het kind, en

dus voor een sterk verhoogde aanmaak van bilirubine bij de pasgeborene.
De standaard behandeling voor geelzucht is fototherapie. Fototherapie werd ontdekt door een verpleegkundige, die had opgemerkt dat pasgeborenen aan de raamkant van de ziekenzaal minder geelzucht leken te hebben dan de baby's aan de muurkant van de zaal. Deze observatie zorgde er uiteindelijk voor dat fototherapie de standaard behandeling werd voor ongeconjugeerde hyperbilirubinemie. Sinds de invoering van fototherapie in de jaren '60 is het aantal patiënten dat hersenbeschadiging oploopt door hyperbilirubinemie enorm afgenomen. Fototherapie is een blauw licht, dat wordt gebruikt om het bilirubinemolecuul minder schadelijk (meer water oplosbaar) te maken. Op deze manier kan dit water oplosbare bilirubine eenvoudig, en dus zonder conjugatie, door de lever worden uitgescheiden. Toch kleven er nog altijd nadelen aan de fototherapie-behandeling. Zo moeten Crigler-Najjar patiënten, aangezien zij een permanent defect hebben van het leverenzym UGT1A1, levenslang worden behandeld. Dit houdt in dat deze patiënten dagelijks, soms tot wel 16 uur per dag, onder een soort zonnebank moeten liggen, die de huid bestraalt met blauw licht. Deze therapie is niet alleen zeer belastend (enorme impact op het sociale leven), maar wordt ook minder effectief naarmate patiënten ouder worden. Door dit verlies aan effectiviteit ontwikkelt circa 25% van de Crigler-Najjar patiënten alsnog hersenschade. Bij neonatale geelzucht is de belasting wat betreft de behandelperiode lager, maar ook aanzienlijk, omdat moeder en kind worden gescheiden. Daarnaast blijven de bilirubinespiegels in het plasma ondanks intensieve fototherapie soms toch fors stijgen. In dat geval wordt vaak gekozen om een wisseltransfusie toe te passen. Bij een wisseltransfusie wordt het bilirubine-rijke bloed van de pasgeborene vervangen door donorbloed met een normaal bilirubinegehalte. Deze vorm van behandeling is ingrijpender dan fototherapie en wordt vanwege de risico's alleen in noodgevallen toegepast.

Het feit dat er nog steeds patiënten hersenbeschadiging oplopen onderstreept de noodzaak voor alternatieve of aanvullende behandelopties.
In het plasma is het meeste (>99%) van het bilirubine gebonden aan plasma eiwitten (voornamelijk aan albumine) en slechts een heel klein deel is vrij (<1%). Op dit moment is het starten en stoppen van de behandeling voor hyperbilirubinemie gebaseerd op de totale bilirubineconcentratie in het plasma. Het is echter bekend dat deze totale bilirubineconcentratie slecht correleert met het optreden van hersenbeschadiging in patiënten. De reden voor de slechte correlatie ligt o.a. aan het feit dat niet het totale bilirubine in het plasma, maar alleen het vrije bilirubine (bilirubine$_{vrij}$) de bloed-hersenbarrière kan passeren en hersenschade kan veroorzaken. Theoretisch zou het verlagen van de bilirubine$_{vrij}$ fractie dus een verlaging van de bilirubineconcentratie in de hersenen kunnen geven. De verlaging van bilirubine$_{vrij}$ in het plasma zou bereikt kunnen worden door bilirubine$_{vrij}$ aan extra albumine te laten binden. In **hoofdstuk 2** hebben wij het effect van fototherapie en het effect van fototherapie in combinatie met albumine toediening op de hoogte van plasma bilirubine$_{vrij}$ concentraties en hersenbilirubine waarden onderzocht. Wij hebben zowel

permanente hyperbilirubinemie (m.a.w. Crigler-Najjar patiënten) als acute hyperbilirubinemie (m.a.w. geelzucht bij pasgeborenen) onderzocht in Gunn ratten. Gunn ratten zijn het meest gebruikte diermodel voor ongeconjugeerde hyperbilirubinemie. Deze ratten hebben, net als patiënten met de ziekte van Crigler-Najjar type I, een genetisch defect in het leverenzym UGT1A1 en kunnen dus geen bilirubine conjugeren en uitscheiden. Daardoor hebben Gunn ratten dan ook een levenslange ongeconjugeerde hyperbilirubinemie.
De resultaten in hoofdstuk 2 laten zien dat albuminetherapie het hypobilirubinemisch effect van fototherapie versterkt in de behandeling van hyperbilirubinemie. In Gunn ratten met permanente geelzucht laat langdurige fototherapie in combinatie met albumine toediening een daling zien in totaal plasma bilirubine-, bilirubine$_{vrij}$- en hersenbilirubine waarden. In Gunn ratten met acute geelzucht zien wij niet alleen dat fototherapie in combinatie met albumine toediening de bilirubine$_{vrij}$ concentraties in het plasma verlaagt, maar zien wij ook dat de bilirubine ophoping in de hersenen geheel voorkomen kan worden. Behandeling met alleen fototherapie kon daarentegen de ophoping van bilirubine in de hersenen bij acute geelzucht niet voorkomen.
Wij denken dat de combinatie van albumine en fototherapie uitermate effectief kan zijn op basis van het volgende principe: eerst verlaagt albumine de bilirubine$_{vrij}$ concentraties in het plasma, waardoor er een nieuw evenwicht kan worden ingesteld tussen weefsels en plasma. In dit geval betekent dit dus dat er een verschuiving plaatsvindt van bilirubine uit de hersenen naar het plasma. Vervolgens kan fototherapie het bilirubine in het plasma omzetten in water oplosbare moleculen en kan de bilirubine worden uitgescheiden via de gal.
Samenvattend geven onze resultaten aanleiding voor een logische vervolgstap in de vorm van een gerandomiseerde klinische studie. In deze studie kan het gebruik van albumine als aanvullende therapie naast fototherapiebehandeling geëvalueerd worden bij patiënten met de ziekte van Crigler-Najjar en pasgeborenen met ernstige ongeconjugeerde hyperbilirubinemie.

Op basis van deze succesvolle en zeer veel belovende effecten van albuminebehandeling, hebben we onze experimenten met albumine toediening voortgezet. In **hoofdstuk 3** hebben we de combinaties van wisseltransfusie, fototherapie en/of albumine toediening bestudeerd. Een wisseltransfusie wordt gezien als een "laatste redmiddel" wanneer bilirubine concentraties extreem hoog zijn, of niet dalen onder fototherapie. Een wisseltransfusie kan echter leiden tot bijvoorbeeld hartproblemen, infecties en er is zelfs een kans op overlijden (in 0,3-2,0% van de gevallen). Onze hypothese is, dat de combinatie van fototherapie met albumine toediening een effectievere (en ook veiligere) behandeling voor ernstige hyperbilirubinemie zou kunnen zijn dan een wisseltransfusie. Allereerst, hebben wij met succes een model voor wisseltransfusies bij ongeconjugeerde hyperbilirubinemie geoptimaliseerd in Gunn ratten. Onze resultaten lieten zien dat dit Gunn rat-wisseltransfusiemodel zeer waardevol kan zijn voor het vergelijken van verschillende behandelcombinaties om hersenschade te voorkomen tijdens acute ernstige ongeconjugeerde hyperbilirubinemie. In ons experiment hebben wij aangetoond dat een

wisseltransfusie de meest effectieve behandeloptie is in de acute situatie. Een wisseltransfusie laat een uur na behandeling een significant sterkere daling van de bilirubine$_{vrij}$ concentraties zien dan de combinatie van fototherapie en albumine. Daarnaast is als acute behandeloptie de combinatie van een wisseltransfusie met fototherapie en/of albumine toediening niet significant beter dan een wisseltransfusie alleen. Als een follow-up behandeling na een wisseltransfusie is de combinatie van fototherapie met albumine toediening wel het meest effectief, waardoor een lage bilirubinespiegel behouden kan worden. Naar ons idee is de beschikbaarheid van dit geoptimaliseerde Gunn rat-wisseltransfusiemodel zeer behulpzaam voor het verder verbeteren van de behandeling voor acute, potentieel neurotoxische, hyperbilirubinemie.

In hoofdstuk 2 en 3 hebben we de effecten van albumine toediening in combinatie met verschillende behandelopties op hersenbilirubine waarden onderzocht. Naast deze biochemische resultaten is het belangrijk om de (schadelijke) effecten van hyperbilirubinemie zelf, en de effecten van de verschillende behandelopties hiervoor, te bepalen met een meer functioneel diagnostische uitkomstmaat voor hersenschade. In **hoofdstuk 4** hebben we voor de eerste keer de effecten van albuminebehandeling op een parameter voor hersenfunctie bepaald, en hebben we dit vergeleken met de hersenbilirubine waarden. Daarnaast hebben we de stap gemaakt van volwassen ratten naar Gunn rat-pups. Aangezien pasgeboren baby's een andere fysiologie en een andere doorlaatbaarheid van de bloed-hersenbarrière hebben dan volwassenen, is het een logische stap om voor het bestuderen van geelzucht en hersenschade bij pasgeborenen te kiezen voor een ratten pup model in plaats van voor een model met volwassen ratten.
Een hoge bilirubine$_{vrij}$ concentratie kan hersenschade veroorzaken en in het bijzonder het gehoorsysteem aantasten. Gebaseerd op deze kwetsbaarheid van het gehoorsysteem voor hyperbilirubinemie kan bilirubine-neurotoxiciteit worden geëvalueerd met zogenoemde "Brainstem Auditory Evoked Potentials (BAEPs)". Brainstem Auditory Evoked Potentials zijn functioneel diagnostische testen die de zenuwgeleiding tussen de gehoorzenuw en de gehoorstructuren in de hersenstam vaststellen.
In onze dierexperimenten in hoofdstuk 4 hebben we op twee manieren acute ongeconjugeerde hyperbilirubinemie veroorzaakt. De eerste manier was met behulp van hemolyse (rode bloedcel afbraak). Door de forse afbraak van rode bloedcellen stijgt de productie van bilirubine. De tweede manier was doormiddel van displacement (vervanging). Bij deze methode hebben wij de ratten pups een medicijn gegeven dat er voor zorgt dat de binding tussen bilirubine en albumine sterk verminderd wordt. Hierdoor zal de bilirubine$_{vrij}$ concentratie dus stijgen. In hoofdstuk 4 hebben we aangetoond dat in zowel het hemolyse als in het displacement model albumine toediening beschermt tegen hersenschade tijdens acute hyperbilirubinemie. Ook vonden wij een verschil tussen de BAEPs metingen (functionele resultaten) en de hersenbilirubine waarden (biochemische resultaten). Dit verschil laat het belang van functionele diagnostische testen zien, in het bijzonder in het veld van ongeconjugeerd (vrij) bilirubine. Samenvattend: onze gunstige

functionele resultaten in ratten pups ondersteunen de mogelijkheid voor eventuele klinische toepasbaarheid van albumine behandeling om hersenschade te voorkomen tijdens ernstige geelzucht bij pasgeborenen.

Wanneer de geelzucht eenmaal aanwezig is zijn er meerdere behandelingen beschikbaar, zoals we hebben laten zien in hoofdstuk 2 en 3. Helaas zijn de preventieve mogelijkheden tegen geelzucht bij de pasgeborene beperkt. Uitscheiding van bilirubine via de ontlasting is de belangrijkste uitscheidingsroute van bilirubine voor het lichaam. In eerdere studies hebben wij aangetoond dat ongeconjugeerde hyperbilirubinemie kan worden behandeld in volwassen ratten, door de bilirubine uitscheiding via de ontlasting te versnellen met behulp van polyethyleenglycol (PEG, een laxeermiddel) of ursodeoxycholaat (UDCA, een galzout). Het preventieve karakter van deze twee middelen voor geelzucht bij de pasgeborene is nog niet bekend. In **hoofdstuk 5** hebben we onderzocht of een preventieve behandeling met PEG of UDCA ongeconjugeerde hyperbilirubinemie kan voorkomen in Gunn rat-pups. Er is gekozen voor pups als model voor geelzucht bij de pasgeborene. Wij hebben dagelijks de transcutane bilirubine concentraties (TcB, bilirubine concentraties in de huid) gemeten vanaf de leeftijd van 1 dag tot en met 21 dagen. Deze "huid-bilirubine-meet-methode" wordt al gebruikt in de kliniek om te voorkomen dat er te vaak en te veel bloed wordt afgenomen bij pasgeborenen. De TcB-methode hebben we voor de toepassing in onze studie eerst gevalideerd voor het gebruik in ratten. Op de leeftijd van 7 dagen zijn wij gestart met de preventieve behandeling via orale toedieningen van of fysiologisch zout (controle groep) of PEG of UDCA. In de controle groep zagen wij dat tijdens het natuurlijke verloop de piek in TcB concentraties ligt op de leeftijd van 15 tot en met 18 dagen. Polyethyleenglycol en UDCA verlagen deze hyperbilirubinemie piek significant vergeleken met de controle groep. In hoofdstuk 5 laten wij in een ratten pup-model het concept zien, dat de behandeling met PEG of UDCA een preventieve mogelijkheid kan zijn voor ernstige geelzucht bij pasgeborenen. De preventie van bilirubine ophoping in het plasma is waarschijnlijk het gevolg van het bevorderen van de uitscheiding van bilirubine via de ontlasting. Onze experimentele resultaten bieden perspectief voor een preventieve strategie tegen geelzucht bij (te vroeg geboren) baby's.

Ten slotte hebben wij de stap van proefdier naar mens gemaakt. Bijna alle te vroeg geboren baby's krijgen neonatale ongeconjugeerde hyperbilirubinemie, en dit kan leiden tot bilirubine-encefalopathie (een achteruitgang in de werking van de hersenen). De huidige behandelrichtlijnen voor ernstige hyperbilirubinemie zijn gebaseerd op totale plasma bilirubineconcentraties en niet op bilirubine$_{vrij}$ concentraties. Zoals hierboven beschreven voorspelt de concentratie bilirubine$_{vrij}$ de kans op bilirubine geïnduceerde hersenschade beter dan het totale bilirubine in plasma. Desondanks wordt bilirubine$_{vrij}$ nog niet routinematig gemeten in de kliniek, gezien de analytische problemen die er zijn om snel betrouwbare bilirubine$_{vrij}$ concentraties te verschaffen. De bilirubine/albumine (B/A) ratio werd aangewezen als een mogelijke alternatieve maatstaf voor het schatten

van de bilirubine$_{vrij}$ concentratie. In **hoofdstuk 6** beschrijven wij voor de eerste keer het verloop van de B/A ratio en de bilirubine$_{vrij}$ concentraties gedurende de eerste 10 levensdagen bij te vroeg geboren baby's. Wij zagen dat de piek in bilirubine$_{vrij}$ concentraties werd bereikt op de vierde levensdag. Dit was één dag nadat de piek concentraties van de B/A ratio en het totale bilirubine in plasma werden gevonden. Wij laten ook een zwakke, maar significante correlatie zien tussen de B/A ratio en de bilirubine$_{vrij}$ concentraties. Met behulp van een multilevel statistiek model, tonen wij aan dat de B/A ratio een significante voorspeller is voor de bilirubine$_{vrij}$ concentratie vanaf de tweede levensdag, maar niet dat de B/A ratio in dit geval een betere voorspeller is dan het totale bilirubine in plasma. Onze resultaten suggereren dat de B/A ratio in vergelijking met het totale plasma bilirubine weinig toegevoegde waarde heeft voor het schatten van de bilirubine$_{vrij}$ concentratie in de kliniek. Wij raden dan ook aan om bilirubine$_{vrij}$ metingen routinematig beschikbaar te maken voor de klinische praktijk, om op deze manier de zorg voor te vroeggeboren baby's met geelzucht meer te kunnen individualiseren.

In het laatste hoofdstuk van dit proefschrift, **hoofdstuk 7**, plaatsen wij onze resultaten in een zowel experimenteel als klinisch kader. Wij houden onze resultaten tegen het licht van de bestaande literatuur. Ten slotte bespreken wij mogelijke dierexperimentele en klinische toepassingen van onze bevindingen voor de toekomst.

In dit proefschrift hebben wij zowel diagnostische als behandelstrategieën ontwikkeld en geoptimaliseerd voor ongeconjugeerde hyperbilirubinemie in Gunn ratten en pasgeborenen. De nieuwe strategieën zouden uiteindelijk kunnen dienen als een alternatieve of aanvullende optie voor de huidige standaard behandeling van hyperbilirubinemie. Ook zouden deze nieuwe strategieën wellicht bilirubine geïnduceerde hersenschade kunnen voorkomen bij patiënten met hyperbilirubinemie. Daaropvolgend denken wij dat onze resultaten zouden kunnen worden gebruikt als basis voor het ontwikkelen van klinische studies bij Crigler-Najjar patiënten en pasgeborenen met ernstige geelzucht.

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Ontspanning naast je werk is zeer belangrijk. Gelukkig heb ik een aantal zeer lieve en begripvolle vrienden om mij heen. Nogmaals wil ik hier mijn excuses aanbieden voor de keren dat ik helaas een afspraak moest afzeggen, omdat ik toch nog even naar mijn ratten moest of er weer te veel samples gemeten moesten worden. Van nu af aan probeer ik mijn leven te beteren.

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Lieve Stellingwerffjes, ook voor jullie een speciale vermelding in dit proefschrift. Wat ben ik blij dat we jullie hebben leren kennen op de camping in Frankrijk in de zomer van 2000. Onze vriendschap is mij heel veel waard. Van het lopen van de Nijmeegse Vierdaagse tot het doen van de Nieuwjaarsduik, niks is te gek voor een Stellingwerff-Schreuder activiteit. Ik kijk uit naar ons 14-jarig jubileum! Jaap en Ina, wat zijn jullie een warme mensen! Bedankt voor jullie interesse in mijn onderzoek, maar ook in mij als persoon. Bij jullie voel ik me altijd welkom, het is bijna een tweede thuis. Albert, bedankt voor de mental support tijdens de marathonvoorbereiding, de gezellige etentjes en goede gesprekken. Geanne, ik moet snel weer eens naar Almere komen. Menno, M.D. Stellingwerff, ik vind het erg leuk dat je mijn toekomstige collega wordt! Wie weet mag je binnenkort als co met mij meelopen. ☺

Het is allemaal begonnen in het studentenhuis aan de Korreweg 101A en nog steeds komen de "Hard-Korries" ieder jaar bij elkaar. Anrik, Marianne, Peter, Jildou, Bettina en Prisca, bedankt voor de geweldige (studenten)tijd en de zeer gezellige reünietjes! Fijn dat jullie altijd zoveel interesse in mijn onderzoek tonen. Het feit dat er inmiddels 2 Korreweg-huwelijken zijn gesloten en 4 Korreweg-baby's (en 1 op komst) zijn geboren, laat wel zien dat dit een zeer vruchtbare vriendschap is. ☺

Ook de tweede generatie Korreweggers wil ik hier bedanken voor de vele gezellige etentjes en goede gesprekken. Marco, niemand zal onze humor ooit begrijpen. Ik koester de herinnering aan het birry-ei en de bunte streusel! Ivo, ik begon als student met een poeponderzoek en eindigde met een promotieonderzoek waarbij ik 's nachts tussen de ratten zat. Ook al vond je dit verbazingwekkend, altijd bleef je met veel belangstelling naar mijn hysterische verhalen luisteren. Astrid, tijdens ons co-schap Tropengeneeskunde in Tanzania zijn we echt vriendinnen geworden. Wat een fantastische ervaring! Ook ben ik erg blij dat Ivo en jij mijn verjaardag nooit zullen vergeten. ☺ Bettina, samen horen wij bij beide Korrie-groepjes. Je bent een lieve en gezellige meid. Ik hoop dat er nog vele Korreweg-reünietjes mogen volgen!

FAMILIE

"Home is where the heart is." – Pliny the Elder

Lieve Eildert en Hermien, vanaf het eerste moment dat ik jullie ontmoette heb ik mij welkom gevoeld bij jullie thuis. Bedankt voor de warme opname in jullie gezin en jullie interesse in mij! Ik hoop dat er nog vele kopjes thee, etentjes en boottochtjes mogen volgen.

Lieve Jeroen en Rob, lieve zwagers, bedankt voor jullie interesse in mijn onderzoek de afgelopen jaren. Jullie zijn een prachtige aanvulling op het "vrouwen-gezin-Schreuder". Ik hoop dat er nog vele Sinterklaasavonden en Kerstdinertjes samen volgen.

Lieve Irene en Madelon, wat ben ik blij met twee van zulke lieve zusjes. De afgelopen jaren hebben jullie steeds weer laten blijken hoe trots jullie op mij zijn. Of het nu om een publicatie, een praatje op een congres, of het uitlopen van de New York Marathon ging, keer op keer hebben jullie in mijn vreugde gedeeld. Nu kan ik eindelijk eens zwart op wit zetten, dat ik ook super trots op jullie ben lieve zussies!

Lieve papa en mama, wat ben ik er trots op dat ik jullie dochter ben! De liefdevolle opvoeding die jullie mij hebben gegeven heeft mij gevormd tot wie ik nu ben. Het gevoel dat ik altijd bij jullie terecht kan, is van onschatbare waarde. Wat heb ik een geluk dat ik uit zo'n warm nest kom. Jullie rotsvaste vertrouwen in mij heeft mij de kracht gegeven om altijd het beste uit mezelf te halen. Bedankt voor jullie onvoorwaardelijke steun en liefde!

"Life is what happens to you, while you're busy making other plans." – John Lennon

Lieve Floris, mijn liefste schat, wie had ooit gedacht dat jij het mooiste "project" van mijn promotieonderzoek zou worden. Jij verrijkt mijn leven, iedere dag weer. Bedankt voor alles lief! Jij haalt het beste in mij naar boven. De ware vind je hooguit één keer in je leven en ik heb dat geluk. Ik kijk uit naar onze toekomst samen en hoop dat al onze dromen werkelijkheid worden. Ik geloof in jou en mij!

LIST OF ABBREVIATIONS

AAP	American Academy of Pediatrics
ABR	auditory brainstem responses
ABC	ATP-binding cassette
Alb	albumin
ANOVA	analysis of variance
APHZ	1-acetyl-2-phenyl-hydrazine
AUC	area under the curve
BAEP	brainstem auditory evoked potentials
B/A ratio	bilirubin/albumin ratio
BBB	blood-brain barrier
B_f	free bilirubin
BIND	bilirubin-induced neurologic dysfunction
BW	birth weight, bodyweight
CNS	central nervous system
CO	carbon monoxide
CYP1A1/2	microsomal mixed-function oxidase 1/2
EEG	electroencephalogram
e.g.	for example
EHC	enterohepatic circulation
ET	exchange transfusion
et al.	and others
FDA	food and drug administration
GA	gestational age
Hb	hemoglobin
HbF	fetal hemoglobin
HO	heme oxygenase
HPLC	high-liquid performance chromatography
HRP	horseradish peroxidase
HSA	human serum albumin
Ht	hematocrit
i.e.	in other words
i.m.	intramuscular
i.p.	intraperitoneal
i.v.	intravenous
IVIG	intravenous immunoglobulin
IWI	interwave-interval

K_a	intrinsic albumin-bilirubin binding affinity constant
LED	light-emitting diodes
MRP	multidrug resistant protein
NS	not significant
OAE	oto-acoustic emissision
OLT	orthotopic liver transplantation
Phz	phenylhydrazine
PEG	polyethylene glycol
PT	phototherapy
R	correlation coefficient
RBC	red blood cell
RCT	randomized controlled trial
RES	reticuloendothelial system
SD	standard deviation
SnMP	tin mesoporphyrin
Sulfa	sulfadimethoxine
TBE	total blood exchange
TcB	transcutaneous bilirubin
TSB	total serum bilirubin
UCB	unconjugated bilirubin
UGT1A1	uridine-diphosphoglucuronosyltransferase
UDCA	ursodeoxycholic acid

BIOGRAFIE

Andrea Bertilde Schreuder werd geboren op 26 juli 1985 te Hardenberg. Zij groeide op in Drachten samen met haar ouders en twee zusjes. Aan het Drachtster Lyceum in Drachten behaalde zij in 2003 haar VWO-diploma met een acht gemiddeld, waardoor zij direct aan de studie Geneeskunde mocht beginnen aan de Rijksuniversiteit in Groningen. Nadat zij zowel haar propedeuse als bachelor *cum laude* had behaald, vertrok zij in jaar 5 van haar studie naar het Medisch Spectrum Twente (MST) in Enschede voor haar senior co-schappen. Aansluitend aan deze co-schappen heeft zij 10 weken in het Igogwe District Hospital in Tanzania gewerkt. Haar semi-arts stage kindergeneeskunde heeft zij eveneens met veel plezier gelopen in het MST in Enschede. Vervolgens keerde zij terug naar Groningen waar zij voor het eerst in aanraking kwam met wetenschappelijk onderzoek bij de afdeling kindergastroenterologie en hepatologie van het Beatrix Kinderziekenhuis, Universitair Medisch Centrum Groningen. Onder begeleiding van dr. Patrick van Rheenen en drs. Els van de Vijver heeft zij haar wetenschappelijke stage gedaan naar fecale biomarkers bij kinderen met inflammatoire darmziekten. Tijdens deze wetenschappelijke stage werd haar interesse voor het doen van onderzoek gewekt en werd zij door haar begeleiders voorgedragen als kandidaat voor een promotieonderzoek.

In oktober 2009 startte Andrea met haar promotieonderzoek op het gebied van ongeconjugeerde hyperbilirubinemie in het laboratorium van de afdeling kindergeneeskunde in het Universitair Medisch Centrum Groningen, onder begeleiding van prof. dr. Henkjan Verkade en dr. Christian Hulzebos. De bevindingen van dit onderzoek staan beschreven in dit proefschrift. Gedurende haar promotietraject heeft Andrea op meerdere nationale en internationale congressen haar werk mogen presenteren. Daarnaast heeft zij onderzoek gedaan op het lab van prof. dr. Steven Shapiro in Richmond, Virginia, Verenigde Staten en op het lab van prof. dr. Libor Vitek in Praag, Tsjechië.
Tijdens haar onderzoeksperiode heeft Andrea de Nijmeegse Vierdaagse gelopen samen met haar vader, heeft zij de Marathon van New York gelopen voor KiKa (Kinderen Kankervrij) en heeft zij deelgenomen aan een World Servants bouwproject in Ethiopië. Daarnaast heeft zij een felbegeerde plek weten te veroveren in het Concilium Hilaricul Pediatricum, de cabaretgroep van de Nederlandse Vereniging Kindergeneeskunde.
Sinds 1 juli 2013 is Andrea werkzaam als arts-assistent kindergeneeskunde in het Medisch Centrum Leeuwarden.

BIOGRAPHY

Andrea Bertilde Schreuder was born on the 26th of July 1985 in Hardenberg, The Netherlands. She grew up in Drachten, together with her parents and two sisters. In 2003 she graduated from secondary school at the Drachtster Lyceum in Drachten. In the same year she started studying Medicine at the University of Groningen. After finishing her Bachelor *cum laude*, she went to the Medisch Spectrum Twente (MST) in Enschede, for her internships. After this year of internships, she worked for 10 weeks in the Igogwe District Hospital in Tanzania. She did her final internship in Pediatrics also in the MST in Enschede with a lot of enthusiasm. Subsequently, she returned to Groningen where she came in touch with medical research for the first time, at the department of Pediatric Gastroenterology and Hepatology of the Beatrix Children's Hospital, University Medical Center Groningen. Under supervision of dr. Patrick van Rheenen and drs. Els van de Vijver she performed her Master thesis about fecal biomarkers in children with inflammatory bowel disease. During this period her interest for clinical research was born, and she was put forward as a PhD-candidate by her supervisors.

In October 2009 Andrea started her PhD-project in the field of unconjugated hyperbilirubinemia in the Laboratory of Pediatrics at the University Medical Center in Groningen, under supervision of prof. dr. Henkjan Verkade en dr. Christian Hulzebos. The results obtained in this research-project are described in this thesis. Andrea further developed her skills as a researcher by presenting her work at several national and international conferences. Also, she worked in the laboratory of prof. dr. Steven Shapiro in Richmond, Virginia, USA and in the laboratory of prof. dr. Libor Vitek in Prague, Czech Republic.

During her PhD-project Andrea walked the Nijmeegse Vierdaagse (4 days of 40 km walking) together with her dad, she ran the New York Marathon for KiKa (Children Cancer Free Foundation), and she went to Ethiopia for a building-project of World Servants (a welfare organization for underdeveloped countries). Finally, she acquired a coveted position in the Concilium Hilaricul Pediatricum, the cabaret group of the Nederlandse Vereniging Kindergeneeskunde (Dutch Association of Pediatrics).

Since the 1st of July 2013, Andrea is working as a physician at the department of Pediatrics in the Medical Center of Leeuwarden.

LIST OF PUBLICATIONS

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